CHALMERS

Pre-history of *Listeria monocytogenes* affecting the lag phase and genetic response on cold smoked salmon

Master of Science Thesis in the Master Degree Program Biotechnology

SUSANNE DJURLE

Department of Chemical and Biological Engineering
Division of Food Science
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden, 2012

Pre-history of *Listeria monocytogenes* affecting the lag phase and genetic response on cold smoked salmon

Master thesis performed at SIK – The Swedish Institute for Food and Biotechnology

SUSANNE C. DJURLE

Supervisor:
Maria Lövenklev
Project leader
SIK – The Swedish Institute for Food and Biotechnology

Examiner:
Thomas Andlid
Associate professor
Chalmers University of Technology

Department of Chemical and Biological Engineering
Division of Food science
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden, 2012

Pre-history of *Listeria monocytogenes* affecting the lag phase and genetic response on cold smoked salmon

SUSANNE C. DJURLE

Department of Chemical and Biological Engineering
Division of Food science
Chalmers University of Technology
SE-41296 Göteborg
Sweden

Microbiology and Process Hygiene
SIK – The Swedish Institute for Food and Biotechnology
SE-402 29 Göteborg
Sweden

Available online in Chalmers Publication Library

Pre-history of *Listeria monocytogenes* affecting the lag phase and genetic response on cold smoked salmon

SUSANNE C. DJURLE
Department of Chemical and Biological Engineering
Chalmers University of Technology

Abstract

Listeria monocytogenes is a food pathogen that can grow in biofilms in food processing environments and contaminate food products before or after processing. Of particular concern are ready-to-eat foods, such as cold smoked salmon, since *L. monocytogenes* can grow during storage in refrigeration temperatures and vacuum. Biofilms formed in different temperatures (8°C, 15°C and 37°C) under static conditions or at 15°C under dynamic conditions or static conditions in two different media were used to inoculate cold smoked salmon to study the lag phase duration during a 21-day challenge test. The status of the cells in the different conditions was also studied by evaluating the expression of the general stress gene *sigB* and the virulence gene *prfA* with RT-PCR. The lag phase duration was longest for the challenge test with a biofilm formed in 37°C in static conditions as pre-culture (15.7 days) and decreased with temperature, for the challenge test with a biofilm formed in 8°C under static conditions as pre-culture there was no lag phase. The expression of *sigB* was up-regulated for the biofilm formed in 8°C under static conditions but unchanged for the other conditions compared to planktonic growth in 37°C BHI. The expression of *prfA* was up-regulated for all the tested conditions and greatly up-regulated for the biofilm formed in 8°C under static conditions. This illustrates the importance of pre-culture conditions for the growth characteristics in a challenge test and for *L. monocytogenes* virulence.

Keywords: *Listeria monocytogenes*, biofilm, lag phase, *sigB*, *prfA*, challenge test, cold smoked salmon, ready-to-eat foods, pre-history

Table of Contents

1 Introduction

1.1 Aim and scope of study

The aim of this study was to extend the knowledge about factors in the pre-history of *Listeria monocytogenes* that influence the lag phase duration and the gene expression of the genes *prfA* and *sigB* during a 21-day challenge test on cold smoked salmon. The pre-histories of interest were biofilms formed under different conditions since biofilms are highly relevant in food industries. The conditions used to form biofilms were static conditions at different temperatures and in different media and dynamic conditions.

This study is part of continuous work concerning *L. monocytogenes* and lag phase at SIK – The Swedish Institute for Food and Biotechnology, and preceding this was the master thesis by Camilla Samuelsson "The influence of different stress factors on the lag phase and genetic expression of *Listeria monocytogenes* in cold smoked salmon" (2012).

1.2 Food contamination

The first reported outbreak from *L. monocytogenes* in the US was in 1981 and was traced to coleslaw. Over the last 30 years, *L. monocytogenes* has become a major problem affecting a large number of foods, including cheese, milk, raw vegetables, meat and fish. There are about 2000 cases each year in the US, with 500 fatalities and most European countries have approximately the same incidence rate (0.7cases per 100,000 people) (Montville 2008). Cheese made from unpasteurized milk may contain *L. monocytogenes* since neither cheese manufacturing or ripening kills them, therefore risk groups are recommended to avoid such cheeses. Pasteurization of milk removes *L. monocytogenes* but if post-process contamination occurs it can grow very well (Montville 2008). Several meat and fish products have been related to outbreaks but since this is a diverse group *L. monocytogenes* cannot grow in all meat or fish products (Montville 2008). Some of the most high risk foods are so called ready-to-eat products that are processed to different degrees, packed and then consumed with no additional treatment. One such product is cold smoked salmon, which is the focus of this study. One problematic factor is that *L. monocytogenes* does not affect the taste, scent or appearance of the food (Burall 2012) which makes it hard for consumers to detect it.

Nowadays the ability to detect and trace outbreaks has increased and utilizes computerized databases and DNA fingerprinting. The time from consuming contaminated food to manifestation of symptoms is up to 5 weeks which can make it hard to locate the source but statistical analysis can be made based on the food often eaten by those who got sick and those that did not get ill and that way the source can sometimes be located.

In many European countries, including Sweden, the limit for *L. monocytogenes* in food is 100cfu/g at the end of shelf life (Commission regulation 2073/2005 (2005)). Food products known to have a higher prevalence of *L. monocytogenes* are tested more frequently than foods that previously have not caused any outbreaks. In 2010 the Swedish national food administration made an extensive study of the prevalence of *L. monocytogenes* in ready-to-eat foods in Swedish food stores. The prevalence in cold smoked salmon was 13% and this was an increase from the last study in 2001 but the prevalence of products with a cfu/g above 100 had decreased (Nilsson 2011). If domestic and imported salmon were separated, the domestic only had a prevalence of 8% while the imported had 44% (Nilsson 2011), indicating that the control of the imported must be improved.

Products that are properly heat-treated should be free from *L. monocytogenes* (since *L. monocytogenes* does not survive heating) but post-processing contamination is a major problem and *L. monocytogenes* is therefore found in many processed products. In the ideal case no pathogens would be able to enter into food-processing plants but there are several routes for *L. monocytogenes* to come into the plant, either with the staff on e.g. their clothing or if they carry an infection asymptomatically or on the food itself e.g. by contaminated vegetables or carcasses (Montville 2008).

L. monocytogenes can be persistent in the food-processing environment and the same strain can be found years after the first isolation (Holah 2004). The design of the pipes and other equipment are very important to enable good cleaning and thereby less risk of microbial contamination (Lelièvre 2002). *L. monocytogenes* have been detected in several places in the industrial environment, including floors, drains and in refrigeration rooms (Carpentier 2011), and also on different equipment since it can attach to many surfaces including stainless steel, rubber and glass (Montville 2008). On these surfaces biofilms can be established and this is one explanation for many of the problems with *L. monocytogenes* in industry since bacteria can detach from the biofilm and contaminate food (Midelet 2002). The definition of a biofilm varies slightly between authors but is in principle when free living planktonic cells attach to and grow on a surface, often in an extracellular matrix of molecules from the medium and/or excreted by the bacteria.

In many ecosystems more bacteria can be found in biofilms than as free planktonic cells (Costerton 1995), perhaps as many as 99% of the bacteria and in biofilms they are more resistant to stresses such as disinfectants than as planktonic cells (Potera 1998). The conditions under which the biofilm is formed affect the growth characteristics including the metabolism as well as resistance to disinfectants and recovery after treatment (Simões 2007, Simões 2006, Belessi 2011). It has also been shown that treatment with disinfectants might increase the virulence of the cells surviving the treatment (Rodrigues 2011). Less nutrition in the medium have been indicated to lead to faster adherence of planktonic cells to surfaces (Oh 2007) but the adherence is also strain and temperature dependent (Norwood 2001). Furthermore can the presence of other bacteria either inhibit or promote adhesion of *L. monocytogenes* (Carpentier 2004, Bremer 2001). Several genes have been shown to be involved in the formation of biofilms but not all have known functions (Chang 2012). Biofilms have been studied more intensely in the last years and one bacterium used as model bacteria in biofilm research is *Pseudomonas aeruginosa* and in this genes that are not essential for planktonic growth but are needed for growth in biofilms have been found (Finelli 2003).

1.3 *Listeria monocytogenes*

There are six species in the bacterial genus *Listeria*. *L. monocytogenes* is a human pathogen and the focus of many studies, *Listeria ivanovii* is mostly pathogenic to animals while *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi* are non-pathogenic. If *Listeria* is mentioned by authorities it is reasonable to assume that they mean *L. monocytogenes*.

Listeria monocytogenes is a facultative anaerobe that can be found in a wide range of environments including food soils, on plants and hard surfaces (Montville 2008). *L. monocytogenes* can grow in acidic environment, on food growth have been reported in pH as low as 4.2 (Buchanan 2004) but it can survive in pH below that (Montville 2008). The growth is nevertheless affected by the acids present, e.g. acetic acid are good at inhibiting growth of

listeria (Montville 2008). *L. monocytogenes* can also grow in a large temperature interval, between 0°C to 45°C, but much slower at low temperatures than near the temperature optimum of 37°C (Montville 2008). The optimal water activity (a_w) is 0.97 (Montville 2008) but many strains can grow in an environment with water activity as low as 0.91-0.93 (Buchanan 2004) and survive for prolonged periods at a_w=0.83 (Montville 2008). *L. monocytogenes* is also salt tolerant, it can grow in 10-12% of sodium chloride and survive for long periods at even higher salt concentrations (Montville 2008). All of these characteristics make *L. monocytogenes* a very widespread and persistent bacterium. As Montville and Matthew (2008), states "given the organisms characteristics, it is unrealistic to make all food *Listeria*-free" although this is often desired since the disease caused by *L. monocytogenes* can be severe.

One major problem with *L. monocytogenes* is that it can grow during refrigeration temperatures and in vacuum (Beumer 1996) , and with the increasing use of refrigeration temperature as the major preservative method and consumers demanding "natural" and "minimally processed" food without preservatives or "additives", that could have inhibited the growth *L. monocytogenes* and other pathogens, the problems with *L. monocytogenes* increases (Montville 2008). This also gives *L. monocytogenes* an advantage over other spoilage bacteria that can't grow during refrigeration but dominates in higher temperatures.

Heating food is an effective way to remove *L. monocytogenes*, it dies at temperatures above 50°C but heating to higher temperatures is recommended for total removal. Freezing, on the other hand, is often not an effective way to kill listeria but the effect on the viability depends on the food and the freezing rate.

1.3.1 Listeriosis

All detected *L. monocytogenes* infections in Sweden should be reported to the Swedish Institute for Infectious Disease Control. In the last years more cases than previously have been reported, with a peak in 2009 with 73 cases, corresponding to 0,78 cases per 100 000 citizens (Smittskyddsinstitutet 2012).

L. monocytogenes can cause invasive listeriosis in pregnant women, newborns, elderly and immunocompromised (e.g. by organ transplants, cancer or HIV) adults. In pregnant women the infection often causes abortion of the foetus or renders the baby stillborn, even though the mother might only experience symptoms similar to mild influenza. For the other groups *L. monocytogenes* can induces septicaemia (blood poisoning) or meningitis (inflammation of membranes surrounding the brain) (Smittskyddsinstitutet 2012) and the motility rate for invasive listeriosis victims are 20-25%.

In addition to invasive listeriosis, *L. monocytogenes* might also cause food poisoning in the more traditional sense. Feverish gastroenteritis (with symptoms similar to influenza) develop in otherwise healthy people within 18-27h after consumption of a high dose of *L. monocytogenes* In most cases healthy people does not experience any symptoms if the consumed food only contain a small amount of *L. monocytogenes*. (Montville 2008) Healthy people, as well as healthy animals, can carry *L. monocytogenes* without showing any symptoms of infection as has been shown in studies analysing the feces of healthy people where 2-6% of the samples contained detectable amounts of *L. monocytogenes*. Among these people were both "ordinary" healthy people as well as those handling food or *L. monocytogenes* in their work (Montville 2008).

1.3.2 Virulence and pathogenesis of *L. monocytogenes*

Many pathogens produces toxins or multiply in the blood causing disease in the host but *L. monocytogenes* enter into the host cells and multiplies inside the cell before spreading through the cell membrane into surrounding cells. It is sometimes called a facultative parasite since it can live both outside and inside host cells. In the liver and spleen *L. monocytogenes* is killed by the immune system but surviving cells can also spread through the blood to other parts of the body. The ability of *L. monocytogenes* to pass directly between cells enables passage into the brain or placenta, that are normally more protected than many other organs. This passage is regulated by several genes including inlA, hly, actA and plcB which all enable the pathogenicity of *L. monocytogenes* (Montville 2008).

1.3.3 Genes of interest

One of the characteristics of *L. monocytogenes* is that it can grow in low temperatures and it has been shown that low temperatures activates the transcription of the gene *sigB* (Becker 2000) that encodes the alternative stress sigma factor. This can associate with the core RNA polymerase and thereby changes the transcription levels of other genes (Gandhi 2007). To be able to grow in chill temperatures, *L. monocytogenes* accumulates compatible solutes such as glycine betaine and carnitine (Gandhi 2007) but this accumulation is defect in *sigB* mutants and this implies that *sigB* regulates this accumulation (Becker 2000). *sigB* is activated by several other stress factors such as increased osmolarity and the presence of EDTA in the growth medium and it is thought to organize the cellular response for several physical and chemical stresses (Becker 1998). *sigB* transcription also increases when cells enter into the stationary growth phase (Becker 2000).

For acid tolerance the expression of *sigB* have been shown to be important (Wiedmann 1998) but possible not essential (Ferreira 2003). In virulence studies in mice, mutant *L. monocytogenes* that showed no acid tolerant response had reduced virulence (Marron 1997) while mutants with higher acid tolerance had increased virulence (O'Driscoll 1996) which might links *sigB* to virulence.

Kazmierczak *et al.* (2003) identified genes that are regulated by *sigB* and several of those were stress or virulence genes which makes *sigB* an important regulator of the general stress response of *L. monocytogenes* (Gandhi 2007), but there are variations between serotypes on the impact of *sigB* (Moorhead 2003). One of the genes regulated by *sigB* is *prfA*, but *prfA* is also regulated by two *sigB* independent pathways (Nadon 2002). *prfA* was discovered by Leimeister-Wächter *et al.* (1990) to be a positive transcriptional regulator of the virulence gene listeriolysin (LisA) and the name is for positive regulatory factor of listeriolysin (lisA) production. *prfA* also regulates other virulence genes such as *inlA*, *actA* and *plcA* as well as its own expression (Dramsi 1993, Shetron- Rama 2003, Mengaud 1991). Further indications of the importance of *prfA* is that a *prfA* deletion mutant showed no virulence in a mouse model (Chakraborty 1992) and *prfA* have also been indicated to positively regulate the formation of biofilms (Lemon 2010).

1.4 Growth Phases

In a bacterial cultivation there are three stages, the one most studied and from where such characteristic parameters as growth rate and generation time are taken is the log phase where the number of bacteria are doubled per time unit. After the log phase is the stationary phase that occurs when the growth is limited by for example low amount of nutrition in the medium or too high concentration of a harmful metabolite. In this stage growth and death of bacterium are in equilibrium but if one prolongs the cultivation there will often be a decline in the number of bacteria. Before the log phase is the lag phase, where no or little growth occur and the bacteria are adapting to the new conditions. If you transfer log phase bacterium to a new batch of the same medium and otherwise the same conditions, the lag phase is very short. Several factors can influence the duration of the lag phase, including the change in temperature, medium and growth state. The lag phase has in recent years gained more attention even though sometimes the notion is almost that there are so many factors that could influence the lag phase duration that it is not worth studying. Even though many factors can influence, same is true for many other phenomena that have been more extensively studied and therefore we now know which these factors are and how they influence the outcome. The lag phase duration has been modelled but more studies are needed to evaluate and extend these models to growth on different foods and from different conditions.

1.5 Challenge studies

A challenge study is designed to determine the growth of a pathogen or spoiler on food and is conducted by adding the studied bacteria to the food and then storing the food in appropriate temperature. Samples are taken to monitor the growth and a growth curve is constructed. In many cases the growth has first been evaluated using a model and the challenge test is performed to verify the results. The aim is to have no contamination but challenge tests study if possible contaminations can grow to unacceptable levels in the product during the shelf-life.

Notermans *et al.* (1991) showed that *L. monocytogenes* grown in egg products with 30% sucrose had a long lag phase if the pre-culture had been grown in BHI but a substantially less lag phase if the pre-culture had been in the egg product with 30% sucrose also before inoculation. This demonstrated clearly that not only the properties of the food were important for the growth characteristics but also the pre-culture conditions. Dykes (2003) evaluated the growth of two strains of *L. monocytogenes* on processed meat products after pre-cultures in 4°C, 20°C and 37°C. He showed that a pre-culture in 4°C resulted in a higher concentration and shorter lag phase on certain meat products but that the effect was strain dependent. Also other studies have showed that pre-culturing in lower temperatures give shorter lag phase duration when storage occurs in low temperatures (Dufrenne 1997, Francois 2007). Poimenidou *et al.* 2009 simulated a dairy processing environment and concluded that cells grown in biofilm at 5°C and 20°C and then detached to inoculate pasteurized milk had shorter lag phase than planktonic cells grown in the same medium at the same temperature. Similar results could be seen in a study by Georgnaras *et al.* (2006). Augustin *et al.* (2000) have constructed a model based on several strains to predict the effect of the pre-culture temperature and medium on the lag phase duration. This shows that low temperature generally gives a shorter lag time and is a good model, but additional factors could be added, e.g. growth in biofilms. Challenge tests can also be made on foods that do not support growth to evaluate the survival which can also be dependent on the pre-incubation temperature, as well as storage temperature, with a higher survival if storage temperature and pre-incubation temperature were both low (Gay 1997).

In 2011 the EU Community Reference Laboratory designed a procedure for challenge tests to determine the growth potential of *L. monocytogenes* in ready-to-eat foods and determined that two conditions were to be used for the pre-culture; one for optimal growth at 37°C in a medium supporting optimal growth and one in a temperature close to the product temperature, but nothing about other conditions were stated for the pre-cultures (Beaufort 2011). Since challenge tests earlier have been performed with only 37°C an additional temperature is important but several other factors might influence the state of the cells and therefore their growth on food.

2. Method and material

2.1 Strain and food

The strain used was *Listeria monocytogenes* CCUG 32964 (aka. SIK 564, Scott A) (Culture Collection, University of Göteborg, Sweden) which is a serotype 4b and originally isolated from human. It is used as a control strain in food industry and selected for comparison possibilities with Samuelsson (2012) since this was the strain Samuelsson (2012) used in the challenge studies. The strain is kept at SIK, Gothenburg in -20°C in a Microbank (Pro-Lab, Canada, Toronto) on glass beads in broth. Two beads were transferred to blood agar and incubated for 24 h in 37°C and then kept at 4°C until inoculation of overnight cultures. Colonies from this first plate were transferred to a fresh blood agar plate, only plates less than 6 days old were used for the challenge tests, but only 4 passages from the original plate were allowed in order to avoid mutations.

This strain was chosen, but different strains of *L. monocytogenes* may show different responses to similar conditions (Arguedas-Villa 2010, Barbosa 1994). This means that the challenge tests presented in this report might have given other results with another strain, but since the aim was not to compare strains but different pre-cultures only one stain was used.

For overnight cultures, where cells grow planktonic, Bacto™ Brain Heart Infusion (BHI) (Becton, Dickinson and company, USA, New Jersey) was used. 10ml of BHI was inoculated with *L. monocytogenes* and incubated in 37°C for 19h.

The cold smoked salmon used came from Falkeskog and were purchased in pakages of 200g at ICA Maxi at Grafiska vägen in Gothenburg. The water activity of the salmon was 0.968±0.010 and the pH was 5.68±0.05 (Samuelsson 2012).

2.2 Medium for pre-cultures

For the biofilm formation, slime broth (SB) was used which contains: Glucose (VWR, USA, Pennsylvania), Fructose (VWR, USA, Pennsylvania), Sucrose (Merck, Germany, Darmstadt), Nutrient broth (Oxoid, UK, Hampshire) and “LAB-lemco” powder (Oxoid, UK, Hampshire) prepared according to Wirtanen (1995). The modified salmon slime broth (MSSB) contained 50% SB and 50% “salmon residue” prepared by adding 1 part cold smoked salmon and 2 parts of distilled, autoclaved water in a BagPage®+400 (Intescience, France, Saint Nom) (stomacher bag with a filter, porosity 280 microns), homogenizing in a stomacher (Seward Limited, UK, Wortington) for 40s and transferring the liquid from the stomacher bag to a flask, keeping the salmon pieces on the other side of the filter.

2.3 Enumeration of *L. monocytogenes*

To enumerate *L. monocytogenes* growing on salmon PALCAM (Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol) agar plates were used, made from PALCAM agar base (Oxoid, UK, Hampshire) and PALCAM selective supplement (Oxoid, UK, Hampshire). These plates are selective for *Listeria* but not specifically for *L. monocytogenes.* Colonies of *Listeria* are grey surrounded by a dark zone while other bacteria either can't grow or form light colonies with a yellow zone. A salmon piece was placed in a BagPage®+400 and 9 parts of buffered peptone water (BPW) (Oxoid, UK, Hampshire) was added. The bag was placed in the stomacher and the content homogenised for 40s. 1ml of liquid from the BagPage®+400 was taken and 0.1ml of proper 10-fold dilutions were spread on PALCAM agar plates. The plates were incubated in 37°C for 48h before counting the colonies. For enumeration of *L. monocytogenes* from overnight cultures and medium sterile before the addition of *L. monocytogenes* tryptone soya agar plates (TSA) were used, made from Tryptone soya broth (Oxoid, UK, Hampshire) and Agar bacteriological (Oxoid, UK, Hampshire). These were also incubated in 37°C for 48h. Plates with 20-200 colonies were used to determine the number of colony forming units (cfu) per gram or ml.

2.4 Experimental setup

The starting point for the pre-cultures was the work by Samuelsson (2012), where 40 ml SB with the concentration of approximately 10^6cfu/ml of *L. monocytogenes* was added to small beakers containing three stainless steel coupons (20x20x2mm) in a small rack. These beakers were incubated on gentle shake for either 19h or 46h in 37°C or 15°C, respectively, and the coupons were then used to inoculate the salmon slices. This setup is what is referred to as a pre-culture in static conditions in SB, which in this work was performed for 37°C, 15°C and 8°C, but with 38ml of SB in each beaker. The static conditions were also used for the modified salmon slime broth (MSSB) in 15°C. The last setup for the pre-culture was a biofilm formed under dynamic conditions in a flow cell in 15°C with SB. This was developed during the project and described in section 3.1 where incubations times for all the setups can also be found.

2.4.1 Inoculation to salmon

The incubation to salmon, as in the work by Samuelsson (2012), was made by taking a stainless steel coupon in tweezers and gently shaking it in a small beaker with BPW for 10 seconds, to remove loosely attached bacteria, and then carefully place the coupon on a salmon slice for 60 seconds, and then the other side of the coupon was placed on another salmon slice for 60 seconds. The tweezers were sterilised between each contact with the coupons, and the coupons were carefully cleaned (autoclaved, washed by hand, machine washed and autoclaved) between different pre-cultures.

Before inoculation the salmon slices were prepared by placing each slice (12-15g) in a petridish in a vacuum bag for easy handling and after inoculation the samples were vacuum packed with a multivac and kept at 4°C, since this is the recommended storage temperature. For each challenge test three un-inoculated salmon slices were also stored and sampled at the last day to evaluate the prevalence of *Listeria* in the package.

2.4.2 Pre-study

Except for the pre-cultures in 37°C and 15°C under static conditions in SB, the first step of each challenge test was to design the pre-culture conditions to obtain an initial concentration

of 10^5 cfu/g on the salmon. For 8°C static conditions in SB only the time had to be determined, for 15°C under static conditions in MSSB the time and the medium had to be developed and for 15°C in dynamic conditions in SB, the time, volume of medium, design of the instrumental setup and flow rate had to be determined.

The amount of bacteria on the surfaces of the stainless steel coupon were studied and related to cfu/g on salmon. As when inoculation a salmon slice, the studied coupon was handled with tweezers and gently shaken in a beaker with BPW for 10 seconds. It was then directly placed in a pre-prepared 50ml tube containing 15ml of BPW and six glass beads (diameter 4mm). The tube was violently shaken for 5 seconds, followed by heavy vortexing for 20 seconds and then another 5 seconds of shaking and 20 seconds of vortexing. 1ml was taken from the tube and proper 10-fold dilutions were plated on TSA. cfu/ml liquid or cfu/cm^2 could then be calculated. To relate this concentration to the initial concentration on salmon a few salmon slices were also inoculated when the concentration was thought to be somewhat similar to the desired and thereby a rough relation could be drawn between the concentration on the surface and on salmon. Thereby the amount of salmon used for pre-study could be reduced. The same methodology was used for all the pre-studies. In addition, to determine the amount of bacteria and the relation between dead and alive bacteria, bacterial dying with acridine orange and LIVE/DEAD® BacLight Bacterial Viability Kits (Molecular Probes, Invitrogen detection technologies, USA) was used.

2.4.4 Calculation of lag phase

8-9 sample outtakes were made during 21 days (25 days for 37°C static conditions in SB since the test by Samuelsson (2012) showed a long lag phase duration). All samples were taken in triplicate and the mean logarithm of cfu/g was plotted versus the time of sampling giving a growth curve, where the general behaviour of the growth could be visualised. To determine the lag phase duration the excel ad-in DMfit was used (Institute of Food Research, UK), that is based on the model by Baranyi and Roberts (Baranyi 1994).

2.5 Gene expression

From the stainless steel coupon at the end of pre-culture and from the salmon at day 0, day 1 or 3 and on the last day of the challenge test a sample was also taken for evaluation of the expression of the genes *sigB, prfA* and *16S-rRNA. 16S-rRNA* is a house-keeping gene and supposedly expressed at similar level during shifting conditions and can therefore be used to normalize the expression between different samples. A reference sample is also needed to generate an expression ratio of each gene compared to "normal" conditions.

2.5.1 Isolation of RNA from biofilm pre-culture

For RNA isolation from biofilms the PowerBiofilm™ RNA Isolation Kit (MO BIO, USA, California) was used with the following modifications to the beginning of the protocol: A stainless steel coupon was risen from unattached bacteria just as if it was to be used to inoculate salmon but instead it was placed in a petri dish and 0,5ml of RNAprotect (Qiagen, Germany, Hilden) was dripped upon it and incubated for 5min. This is to protect the RNA from degradation (QIAGEN 2005). The coupon was then swabbed with a sterile cotton swab in three directions and the swab was placed in the PowerBiofilm Bead Tube™ with 350µl of the solution BRF1 with β-mercaptoethanol and 100µl of solution BFR2. The tube was vortexed vigorously and the swab was removed from the tube. The protocol was then followed from step 4 without modifications. The RNA was kept in -80°C until analysis.

2.5.2 Isolation of RNA from salmon

The same method was used for isolation of RNA from salmon and from BHI. The sample for RNA isolation from salmon was taken from the same liquid as for the *L. monocytogenes* concentration determination. 10ml of RNAprotect and 5ml of liquid from the BagPage®+ 400 were taken to a centrifuge tube and vortexed briefly. It was incubated for 5 minutes in room temperature and then centrifuged at 5000xg for 10minutes in 4°C, the liquid was removed and the pellet frozen in -80°C until RNA isolation.

Samuelsson (2012) tried to use RNA PowerSoil® Total RNA isolation kit (MO BIO, USA, California) with a few modifications for isolation of bacterial RNA from salmon but with poor success. Therefore another method was sought and the one used is a modified version of the procedure used by Lövenklev *et al.* (2004), since this method has previously been used to isolate RNA from meat products (Wallin-Carlquist 2010). This uses phenol:chloroform extraction and precipitation by addition of ethanol and NaAc. DNA was digested by adding RDD buffer and DNase I stock solution from the RNase-Free DNase Set (Qiagen, Germany, Hilden) and following steps b)-i) in the Hints and Troubleshooting Guide of the RNA PowerSoil® Instruction Manual (under "Digesting DNA with RNase-Free DNase"). The RNA was then frozen in -80°C until conversion to cDNA. The full protocol for the isolation can be found in Appendix II.

2.5.3 Reverse transcriptase

The absorbance at 260 nm, 280nm and 320nm were measured for the RNA samples with a BioPhotometer (Eppendorf, UK, Hampshire). For the measurements the samples were diluted in DEPC-Treated water (Ambion, USA, Texas) and this was also used as blank. The RNA concentration was calculated from the absorbance at 260nm with a conversion factor of 40 µg/ml.

The RNA was converted to cDNA with iScript™cDNA Synthesis Kit (BIO-RAD, USA, California) in MyCycler™ Thermal Cycler (BIO-RAD, USA, California). 0,07µg of total RNA was used for each sample of 20µl and the protocol from the manufacturer was followed.

2.5.4 RT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was used to evaluate the genetic expression of the genes *sigB, prfA* and *16S-rRNA*. In PCR a template (c)DNA strand is amplified during temperature cycles in a mixture with primers, nucleotides and a DNA-polymerase. The temperature is varied to enable double stand dissociation, primer annealing and extension of the template in each cycle. In real time PCR the amplification is monitored for example by fluorescent dye that binds to all double strand DNA and the increase of fluorescence correspond to the increase in DNA concentration. The fluorescence/DNA concentration reaches a threshold value after a number of cycles which gives the Ct-value that can be used to quantify the initial concentration. SsoFast™ EvaGreen® Supermix with lox ROX (BIO-RAD, USA, California) was used according to the manufacturer protocol in the 7500 Real Time PCR System (Applied Biosystems, California, Foster City). 2µl of the product from the reverse transcriptase reaction, 1µl of the forward primer and 1µl of the reverse primer, both corresponding to 500nM primer in the final solution, 10µl of SsoFast EvaGreen Supermix (containing DNA-polymeras and fluorescent dye) and 6µl of DNase/RNase-free water was used for a reaction volume of 20µl.

The specific primers bought by Samuelsson (2012) from Invitrogen (USA, New York) for *sigB, prfA* and *16S-rRNA* were used and these are presented in table 1.

Table 1: Primers for the real-time PCR

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
sigB[1]	GCCGCTTACCAAGAAAATGG	AATATTTTCGGGCGATGGAC
prfA[2]	CTATTTGCGGTCAACTTTTAATCCT	CCTAACTCCTGCATTGTTAAATTATCC
16S-rRNA[1]	GATGCATAGCCGACCTGAGA	TGCTCCGTCAGACTTTCGTC

[1]Reference for primers for *16S rRNA* and *sigB* (van der Veen 2010)
[2]Reference for primers for *prfA* (Olesen 2009)

Expression ratios for *sigB* and *prfA* were then calculated from the Ct-values using the equation by Pfaffl *et al.* (2001) (equation 1) and the efficiency of the assays for the different genes from Samuelsson (2012), presented in table 2. The purity of the PCR product was also evaluated using dissociation curves ran at the end of each RT-PCR.

Table 2: Efficiency of the gene assay

Gene	Efficiency
sigB	2.18
prfA	1.88
16S-rRNA	1.89

$$\text{ratio} = \frac{E_{\text{geneX}}^{C_t(\text{control, geneX}) - C_t(\text{sample, geneX})}}{E_{\text{ref gene}}^{C_t(\text{control, ref gene}) - C_t(\text{sample, ref gene})}} \qquad \text{(Equation 1)}$$

3. Results

3.1 Conditions used for the challenge tests

The experimental setup for the dynamic conditions in the flow cell can be seen in figure 1 and the flow rate was determined to 27ml/min, measured and averaged over 10min. The stainless steel coupons for the flow cell had slightly different dimensions (18x25x2mm) than the ones used for static conditions and 21 of these were placed in seven slits in the flow cell, parallel to the flow direction. To compensate for the increased volume needed in the circulating system a total of 400ml of SB was used with an initial concentration of approximately 10^6cfu/ml of *L. monocytogenes*. The pre-culture was run for 68h before inoculation of salmon. For the 15°C static conditions in modified medium, the medium described under 2.2 was used (MSSB) and the pre-culture was incubated almost the same time as the 15°C static conditions SB, 48h (vs. 46h). The pre-culture in 8°C static conditions in SB was run for 140h before inoculation to salmon.

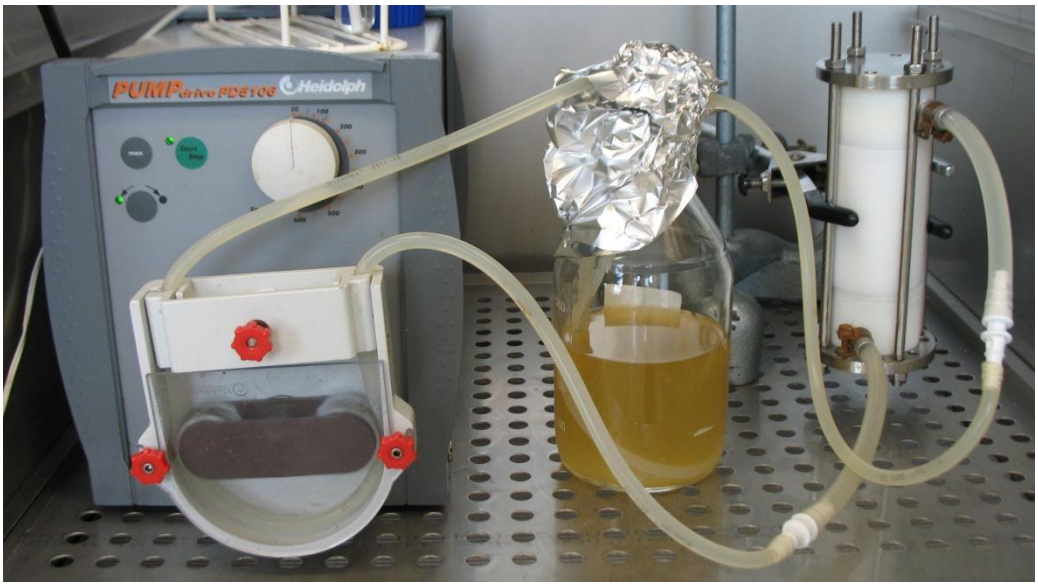


Figure 1: Experimantal setup of the flow cell

3.2 Plating on PALCAM

Injured and stressed cells can sometimes overcome the injury and grow if the conditions are favourable as on nonselective agar but not if they are put on selective agar (Montville 2008). Since PALCAM is selective this might be a concern and some platings on TSA were made parallel with the plating on PALCAM. The cfu/g calculation gave a little higher values on TSA but the difference seemed less at the end of the challenge test (data not shown). This were however not done for all the conditions and no conclusions will be drawn from this except that the actual cfu/g on food might be higher than plating on PALCAM might show, due to stressed bacteria on the food.

3.3 Biofilm in static conditions at different temperatures

3.3.1 Static conditions 37°C

The challenge test with a pre-culture of biofilm formed in 37°C static conditions in SB has been made in two replicates, one by me and one by Samuelsson (2012). As seen in figure 2 these replicates did not give the same growth curves but the combined picture, figure 3, will

be used since this gives a more representative result. All the growth curves presented are based on plating on PALCAM agar and shows means of the triplicates at the sampling time and the error bars are the standard deviation between the samples.

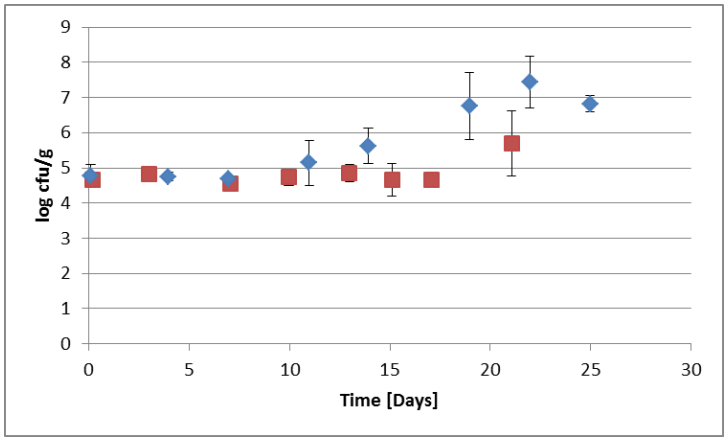


Figure 2: Two replicates of 37°C static conditions in SB

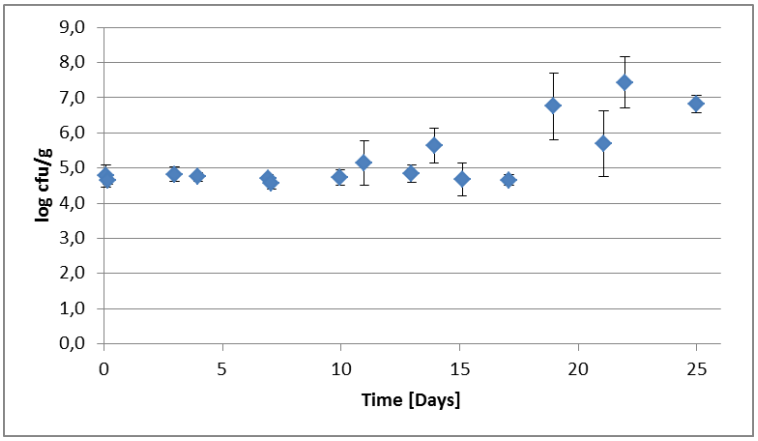


Figure 3: Both replicates of 37°C static conditions in SB

DMfit was used to calculate the lag phase from all values in the two replicates and generated the fit seen in figure 4 and a lag phase duration of 15.65 days.

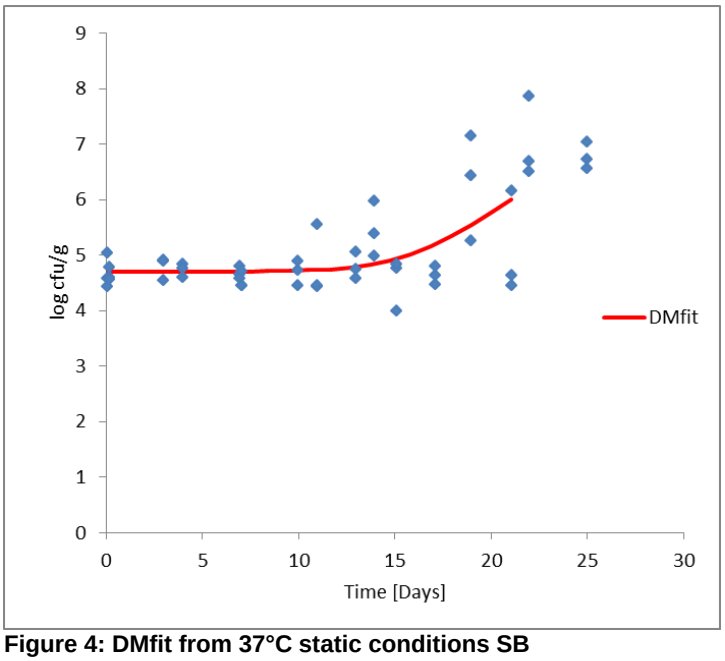


Figure 4: DMfit from 37°C static conditions SB

The growth curve from the pre-culture of biofilm in 37°C static conditions in SB can be compared with the growth curve from an overnight culture as pre-culture, a "standard" pre-culture, 19h in 37°C in BHI where *L. monocytogenes* are grown planktonic. Figure 5 shows this comparison and as seen the initial concentration was lower for the challenge test where the pre-culture was planktionc cells in BHI, but still one can see that the lag phase duration is shorter for the pre-culture in BHI than the biofilm in 37°C static conditions in SB.

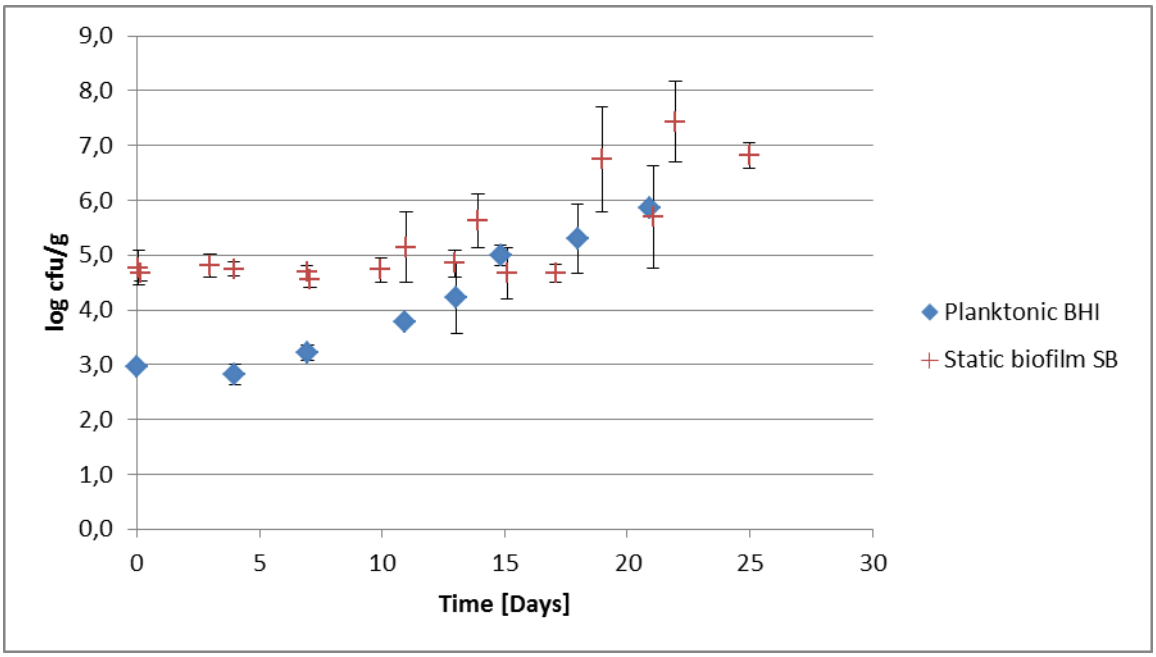


Figure 5: Growth curves of pre-culture 37°C BHI and 37°C biofilm in static conditions in SB

3.3.2 Static conditions 15°C

The challenge test with a pre-culture of biofilm formed in 15°C static conditions in SB was also made by both me and Samuelsson (2012). These were quite similar, figure 6, which makes the combined graph, figure 7, quite coherent.

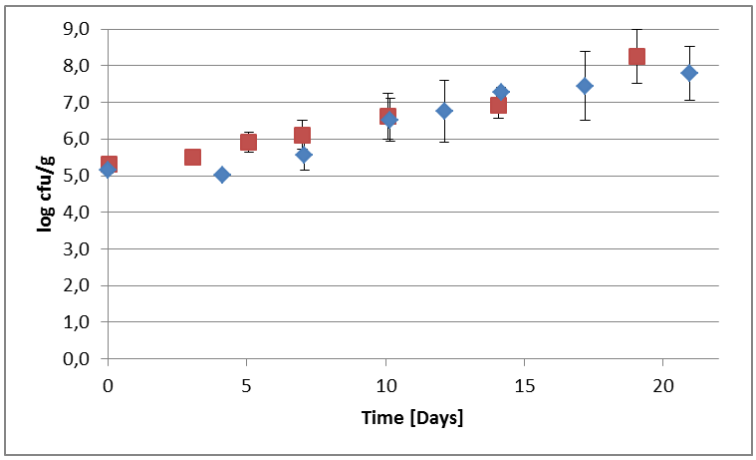


Figure 6: Two replicates of 15°C static conditions in SB

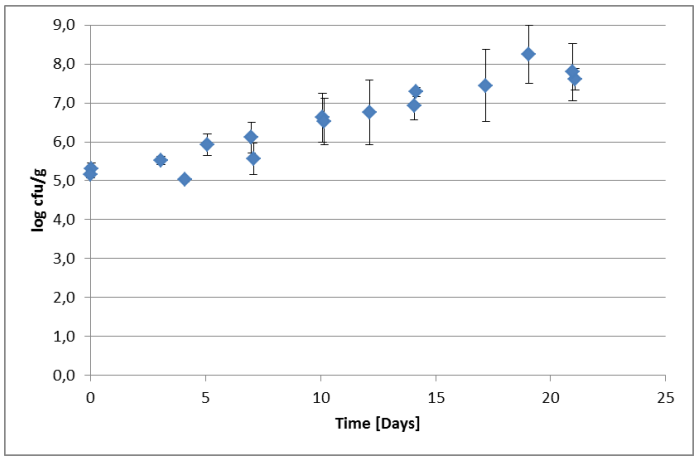


Figure 7: Both replicates of 15°C static conditions in SB

The quite big differences between the samples at each sample point inhibited DMfit from finding a lag phase with all values, therefore the mean values for each point were used instead and DMfit generated the fit seen in figure 8 and a lag phase duration of 3.76 days.

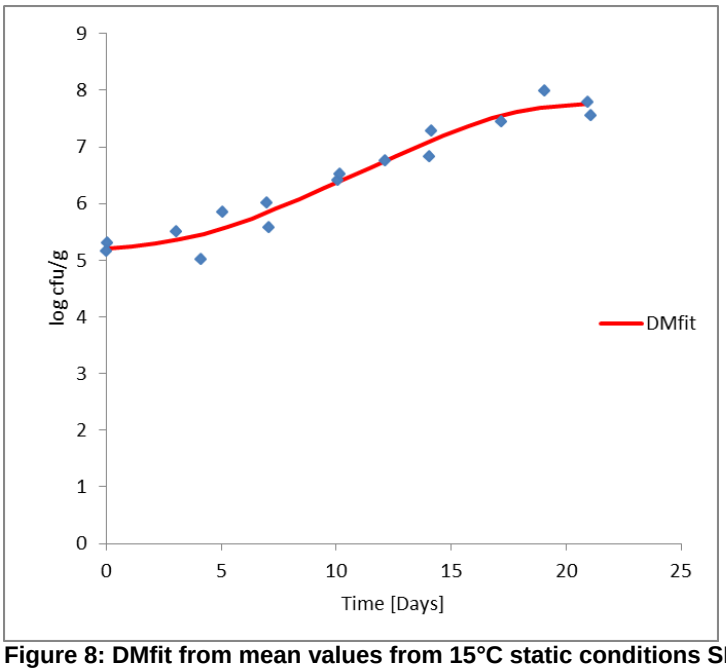


Figure 8: DMfit from mean values from 15°C static conditions SB

3.3.3 Static conditions 8°C

The challenge test with a pre-culture of biofilm formed in 8°C static conditions in SB can be seen in figure 9.

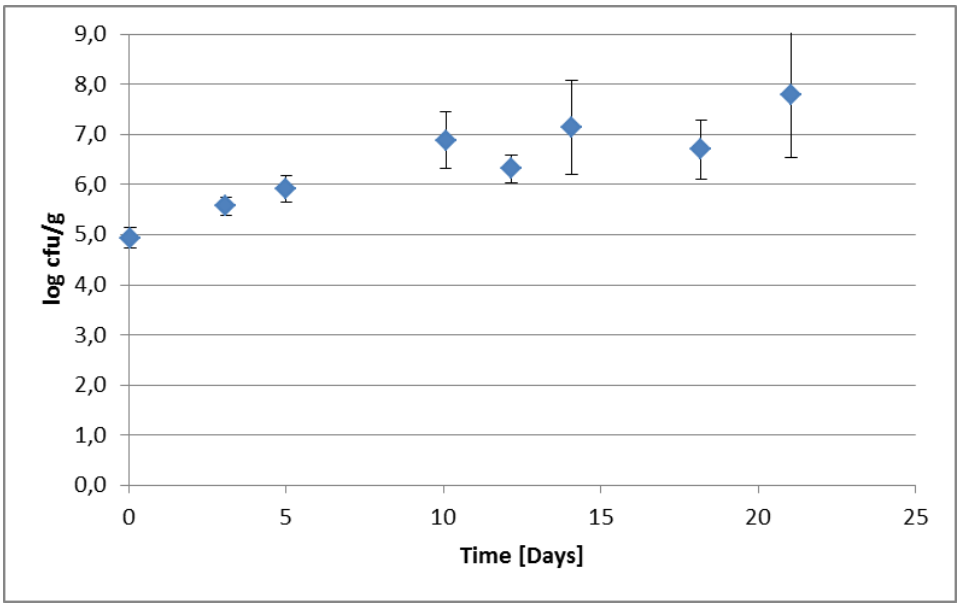


Figure 9: Growth curves of pre-culture in 8°C biofilm static conditions in SB

No visible lag phase can be seen in the growth curve and DMfit could not find one but generated the fit seen in figure 10 representing direct entry into the exponential growth phase.

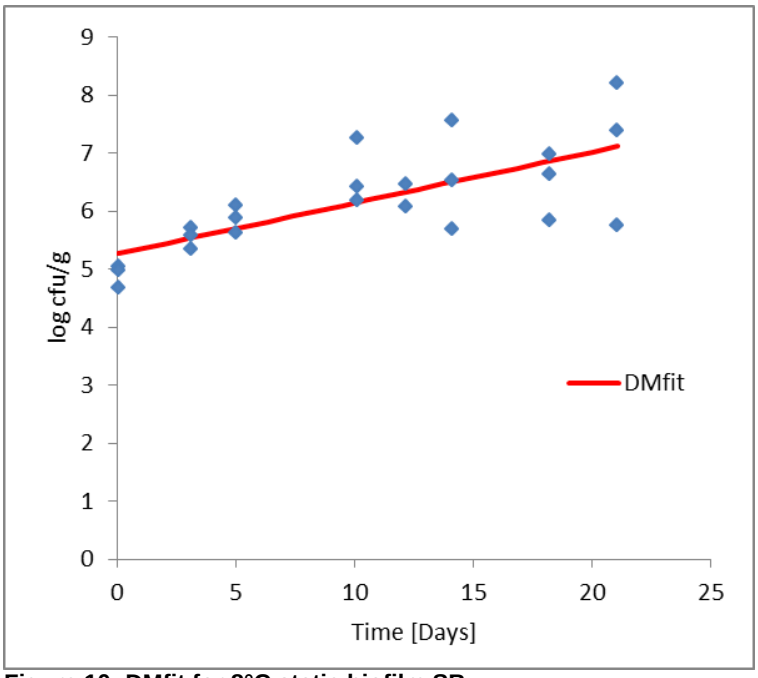


Figure 10: DMfit for 8°C static biofilm SB

3.3.4 Summary of static conditions in different temperatures

The difference in growth curves of the different temperatures can be seen in figure 11. 15°C and 8°C static conditions in SB are quite similar while 37°C is separated by a pronounced lag phase. It can also be seen that the standard deviation is greater at the end of the challenge tests for all temperatures.

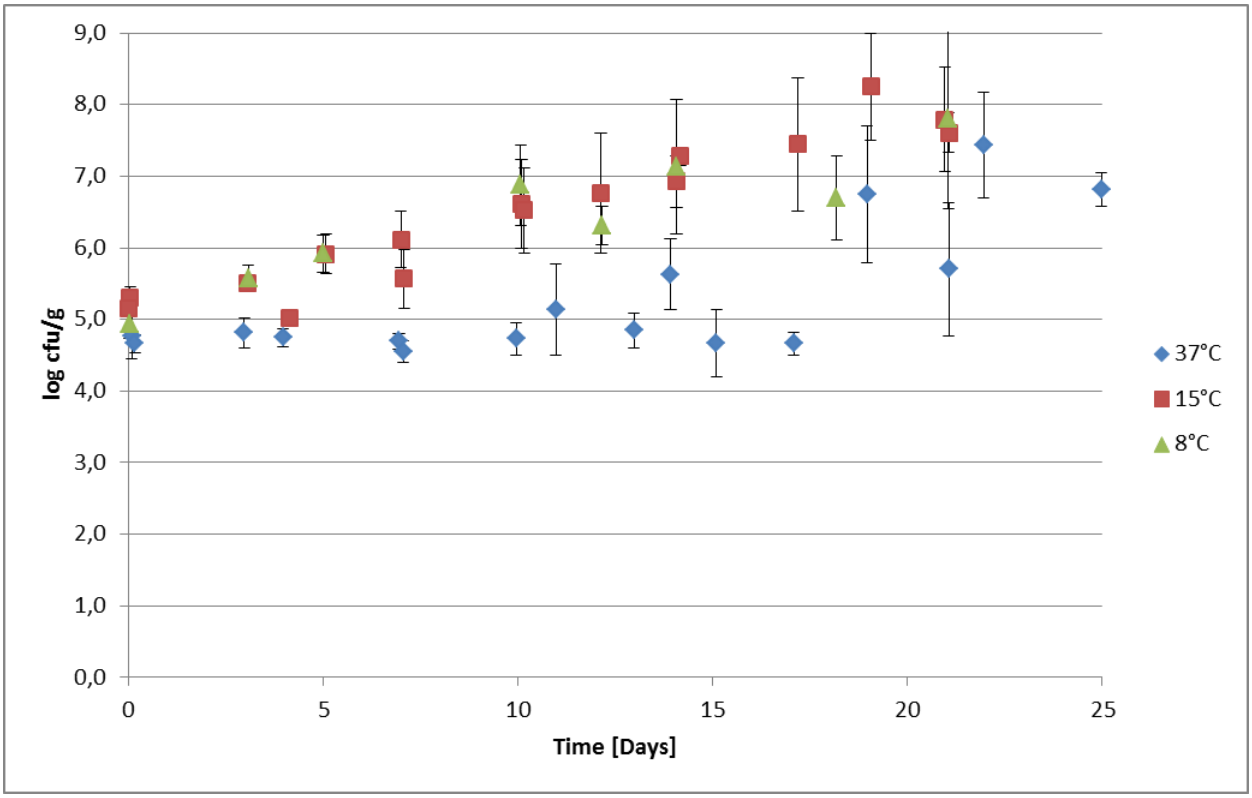


Figure 11: Growth curves for pre-culture of biofilm formed in static conditions at different temperatures in SB

3.4 Biofilm in different conditions in 15°

The growth curve from the challenge test with a pre-culture of biofilm formed in 15°C in dynamic conditions in SB can be seen in figure 12 and the growth curve from the 15°C static conditions in MSSB can be seen in figure 13.

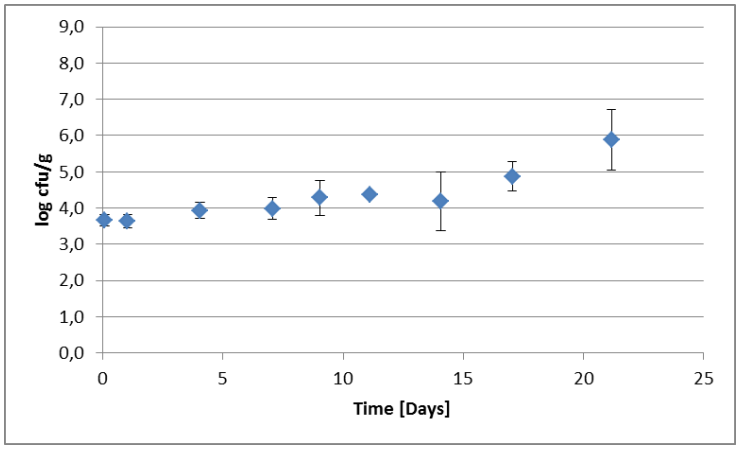


Figure 12: Growth curve from 15°C dynamic biofilm in SB

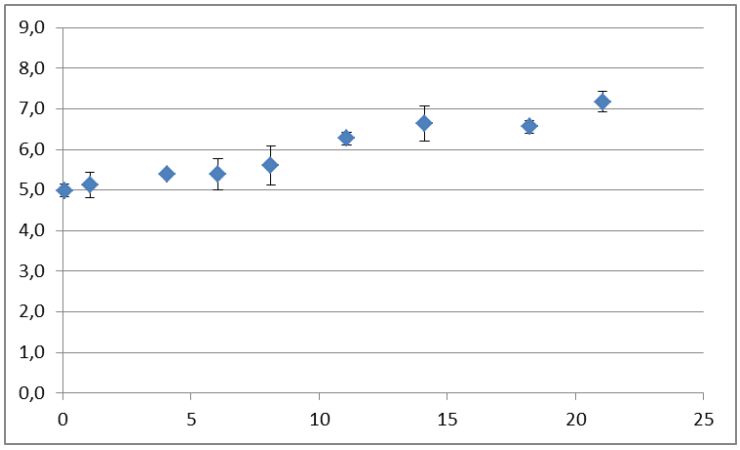


Figure 13: Growth curve from 15°C static biofilm in MSSB

If studying the growth curves for 15°C static conditions in MSSB and 15°C dynamic conditions in SB there seems to be a lag phase but the analysis by DMfit does not generate one, regardless if all values or only the means are used. Therefore a visual estimation of the lag phase duration for these were made and the lag phase duration from 15°C dynamic conditions in SB was approximated to 7 days while from 15°C static conditions in MSSB it was approximated to 5 days.

A comparison between all challenge tests in 15°C in different conditions can be seen in figure 14. The challenge test with pre-culture from the flow cell had a lower initial concentration, since a higher initial concentration could not be reach in the setup.

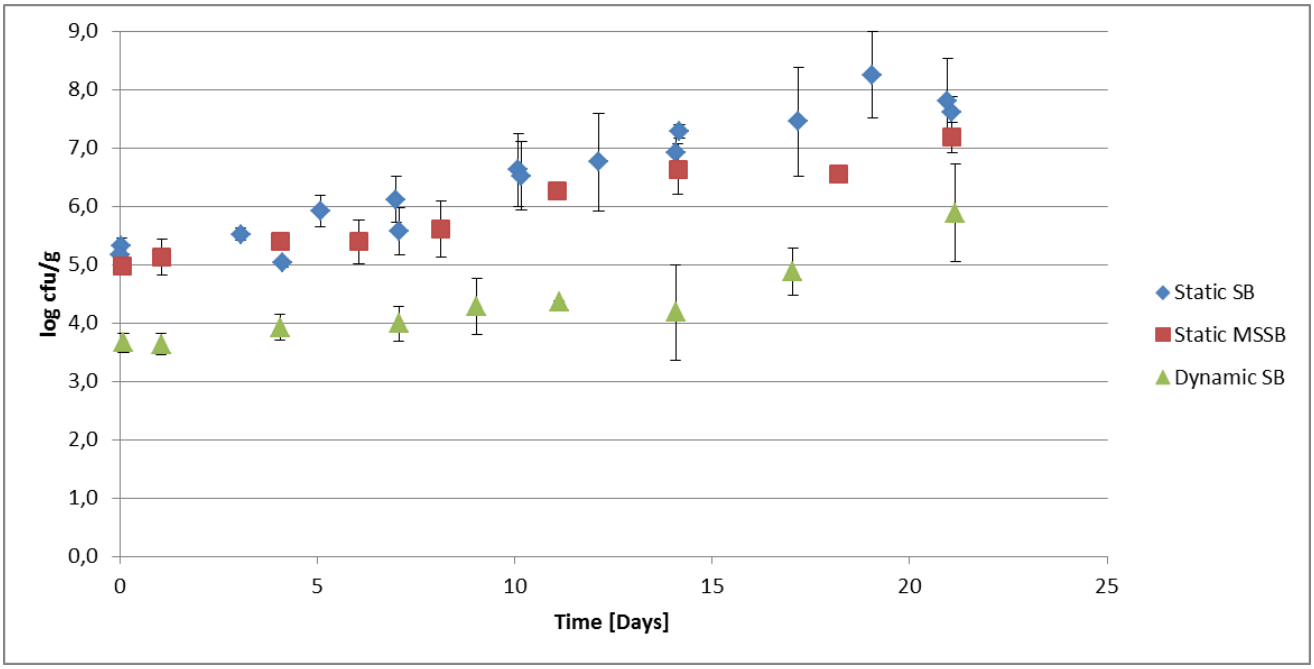


Figure 14: Growth curves from different conditions for the pre-culture, static conditions in SB or MSSB and dynamic conditions in SB, all in 15°C.

3.5 Lag phase duration

The lag phase duration and growth rates from all the challenge tests are summarised in table 3 and visualised in figure 15. Additional curves generated by DMfit can be found in Appendix I.

Table 3: Lag phase duration and growth rate for all challenge tests

Pre-culture	Lag phase duration (days)	Growth rate ($days^{-1}$)
37°C static SB	15.7	0.240
15°C static SB	3.8	0.127
8°C static SB	0	0.088
15°C dynamic SB	7[1]	0.069
15°C static MSSB	5[1]	0.101
37°C planktonic BHI[2]	6.8	0.240

[1]Visual approximation
[2]Samuelsson (2012)

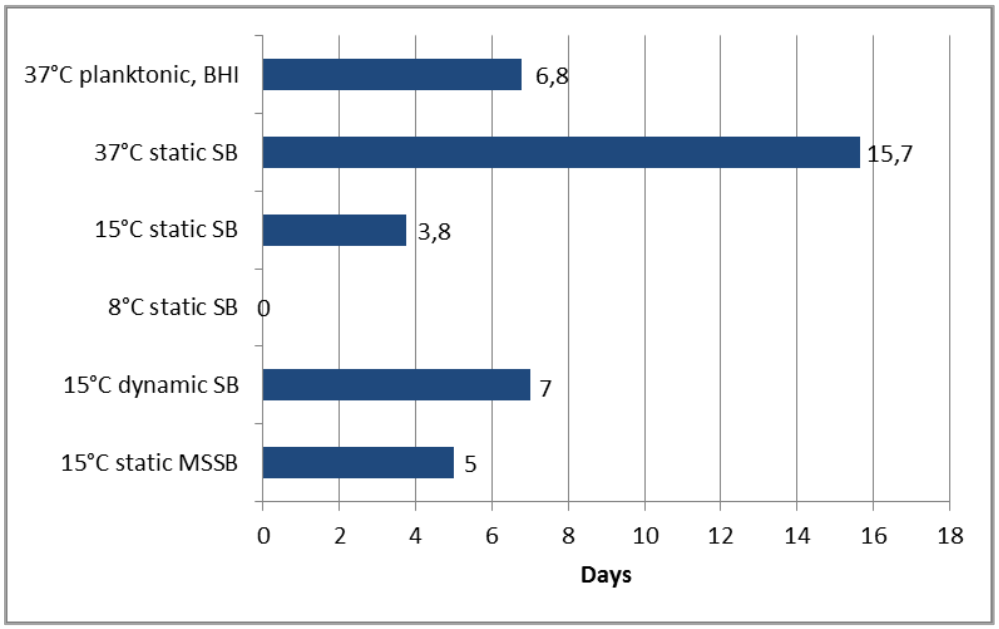


Figure 15: Lag phase duration for all the challenge tests

To obtain a visualisation of the differences from a standard pre-culture, ie. 37°C planktonic in BHI, the lag phase duration for each pre-culture was normalised to 37°C planktonic BHI and presented in figure 16. This shows that 37°C static conditions in SB have more than 2 times longer lag phase duration, 15°C dynamic conditions in SB has a slightly longer while 15°C static conditions in SB and in MSSB have a little shorter as well as 8°C static conditions in SB since it has no lag phase.

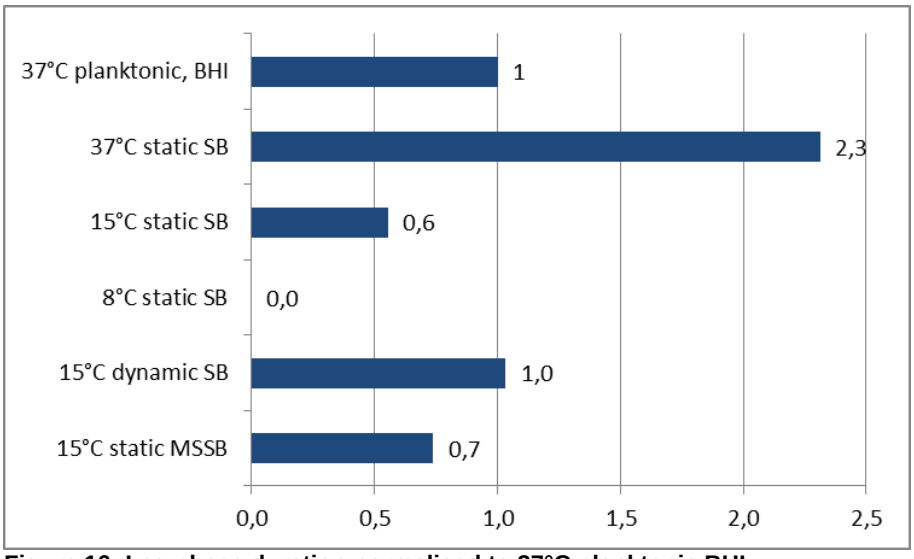


Figure 16: Lag phase duration normalised to 37°C planktonic BHI

3.6 Gene expression

3.6.1 RNA concentration

The RNA concentrations and the A_{260}/A_{280} and A_{260}/A_{230} ratios of the isolated samples are shown in table 4.

Table 4: RNA concentration and purity indications

Pre-culture	Sample	RNA concentration [µg/ml]	A_{260}/A_{280}	A_{260}/A_{230}
37°C static SB	Surface/pre-culture	14	1.27	0.57
	Salmon day 0	27	1.63	2.2
	Salmon day 1	25	1.65	1.81
	Salmon day 25	106	1.67	1.87
15°C static SB	Surface/pre-culture	17	1.27	0.51
	Salmon day 0	697	1.93	2.33
	Salmon day 3	374	1.77	2.09
	Salmon day 21	472	1.81	2.26
8°C static SB	Surface/pre-culture	12	1.4	0.47
	Salmon day 0	238	1.7	2.08
	Salmon day 3	860	1.98	2.43
	Salmon day 21	117	1.63	1.84
15°C Dynamic SB	Surface/pre-culture	16	1.33	0.38
	Salmon day 0	40	1.57	1.3
	Salmon day 1	36	1.63	2.03
	Salmon day 21	291	1.76	2.46
15°C static MSSM	Surface/pre-culture	15	1.29	0.47
	Salmon day 0	58	1.59	1.33
	Salmon day 1	13	1.34	0.59
	Salmon day 21	243	1.77	2.46
37°C planktonic BHI	Liquid/Pre-culture	2430	1.89	2.11
	Salmon day 21	1307	2.07	2.51

For pure RNA A_{260}/A_{280} should be ~2 and A_{260}/A_{230} should be 2-2.2 and deviations from these values indicate different kinds of contaminations.

3.6.2 Ct-values from RT-PCR

The mean Ct-values for all the samples from the RT-PCR are presented in table 5. Three important observations can be made from this:

- No *prfA* could be detected from any of the samples from salmon.
- The mean Ct-value for *sigB* of no template control (NTC), 35.1, is close to many of the samples. Therefore it is hard to say if there is any RNA in those samples and therefore only the samples from pre-culture can be considered. The same tendency can be seen for *16S-rRNA* from salmon.
- The mean Ct-values for *16S-rRNA* for the pre-cultures range between 8.2-20.3 cycles. This is a limitation but will be considered acceptable for analysing the expression ratio with equation 1 (by Pfaffl (2001)).

Table 5: Mean Ct-values for all genes and samples

Pre-culture	Sample	*sigB*	*prfA*	*16S-rRNA*
37°C static SB	Surface/pre-culture	27.9	26.0	13.5
	Salmon day 0	34.1	-	31.2
	Salmon day 1	34.5	-	32.7
	Salmon day 25	34.8	-	33.6
15°C static SB	Surface/pre-culture	27.9	30.0	13.8
	Salmon day 0	33.7	-	33.2
	Salmon day 3	33.6	-	32.2
	Salmon day 21	34.1	-	23.3
8°C static SB	Surface/pre-culture	27.6	27.6	20.3
	Salmon day 0	33.3	-	22.1
	Salmon day 3	34.5	-	29.3
	Salmon day 21	34.0	-	32.3
15°C dynamic SB	Surface/pre-culture	28.6	26.3	13.8
	Salmon day 0	34.1	-	31.2
	Salmon day 1	34.4	-	33.0
	Salmon day 21	34.6	-	26.5
15°C static MSSB	Surface/pre-culture	29.9	28.4	16.2
	Salmon day 0	34.6	-	31.1
	Salmon day 1	35.8	-	31.9
	Salmon day 21	35.4	-	25.4
37°C planktonic BHI	Liquid/Pre-culture	22.7	27.4	8.1
	Salmon day 21	34.9	-	24.9
	NTC	34.4	-	33.5

The Ct-values that are analysed for relative expression are summarised in table 6.

Table 6: Mean Ct-values for the pre-cultures

Sample/gene	*sigB*	*prfA*	*16S-rRNA*
37°C planktonic BHI pre-culture	22.7	27.4	8.1
37°C static SB pre-culture	27.9	26.0	13.5
15°C static SB pre-culture	27.9	30.0	13.8
8°C static SB pre-culture	27.6	27.6	20.3
15°C dynamic SB pre-culture	28.6	26.3	13.8
15°C static MSSB pre-culture	29.9	28.4	16.2

3.6.3 Dissociation curves

The dissociation curves for *sigB* and *prfA* in the RT-PCR for the samples isolated from salmon did not have the desired single peak (figure 19) but instead several peaks, figure 17, or no peak, figure 18. This indicates a non-pure RT-PCR product or no RT-PCR product and also promotes the decision not to analyse these samples any further. The samples originated from pre-culture had a single peak as in figure 19 for all genes, and also all *16S-rRNA* analysis had a single peak, indicating a pure PCR product.

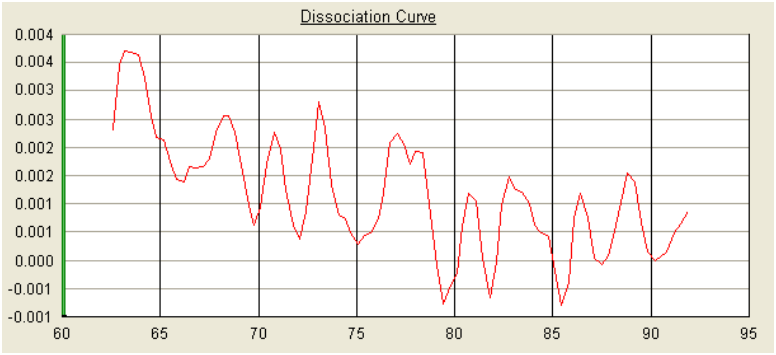


Figure 17: Example of dissociation curve with no/un-pure PCR product

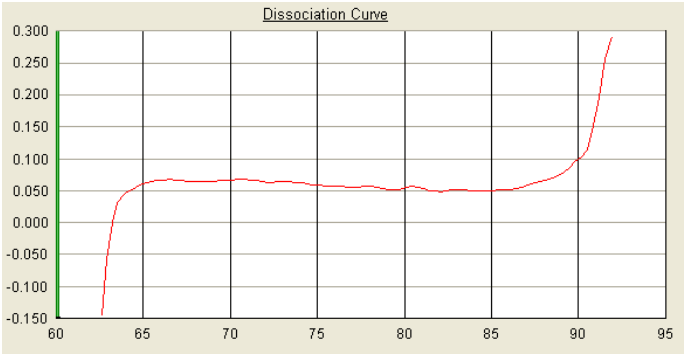


Figure 18: Example of dissociation curve with no/un-pure PCR product

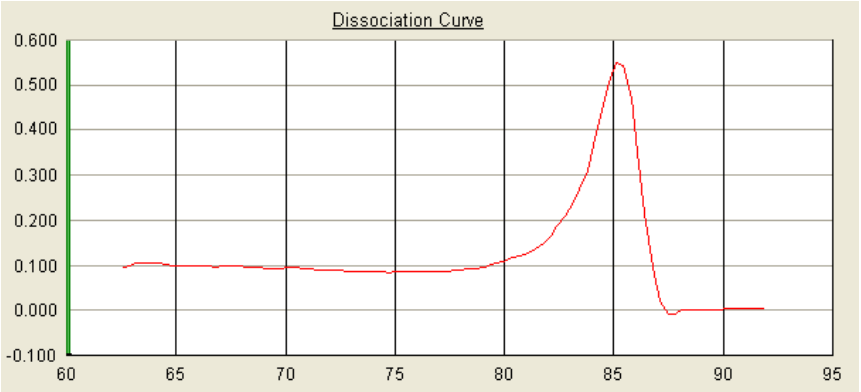


Figure 19: Example of dissociation curve with pure PCR product

3.6.4 Relative expression

L. monocytogenes grown planktonic at 37°C in BHI is used as a standard sample to calculate relative expression and the expression ratio can be seen in table 7 and visualised in figure 20-21.

Table 7: Expression ratio normalised to planktonic growth 37°C in BHI

Gene/sample	37°C planktonic BHI pre-culture	37°C static SB pre-culture	15°C static SB pre-culture	8°C static SB pre-culture	15°C dynamic SB pre-culture	15°C static MSSB pre-culture
sigB	1.00	0.52	0.61	50.84	0.52	1.13
prfA	1.00	72.27	6.91	2050.54	85.86	86.18

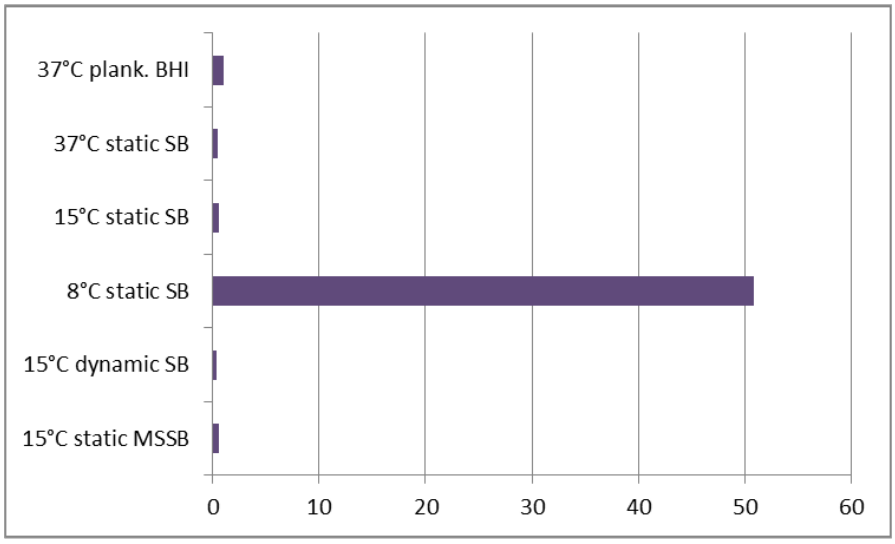


Figure 20: Expression ratio of *sigB* normalised to 37°C planktonic BHI

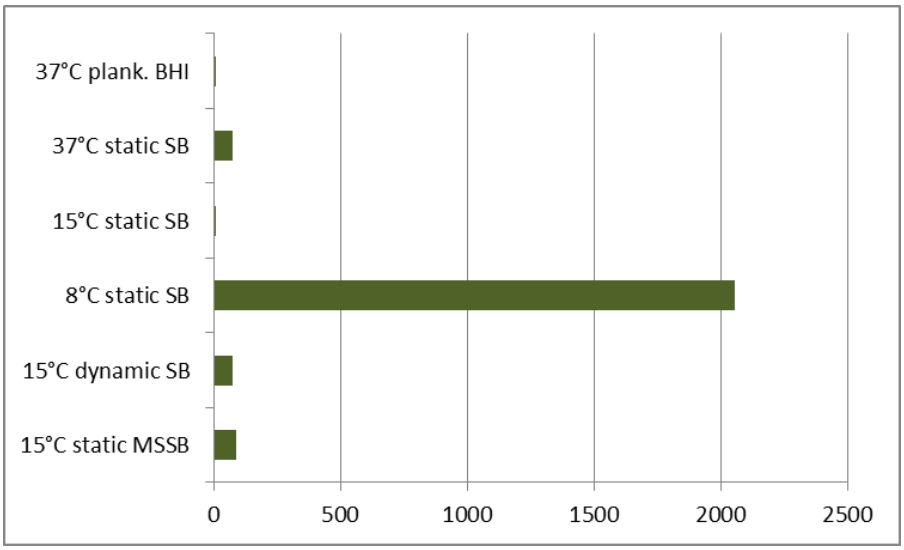


Figure 21: Expression ratio of *prfA* normalised to 37°C planktonic BHI

In these figures it is clear that growth in a static condition biofilm at 8°C up-regulates both *sigB* and *prfA*, but to get a better picture of the other conditions, figure 22-23 shows the same as figure 20-21 but without 8°C static conditions in SB.

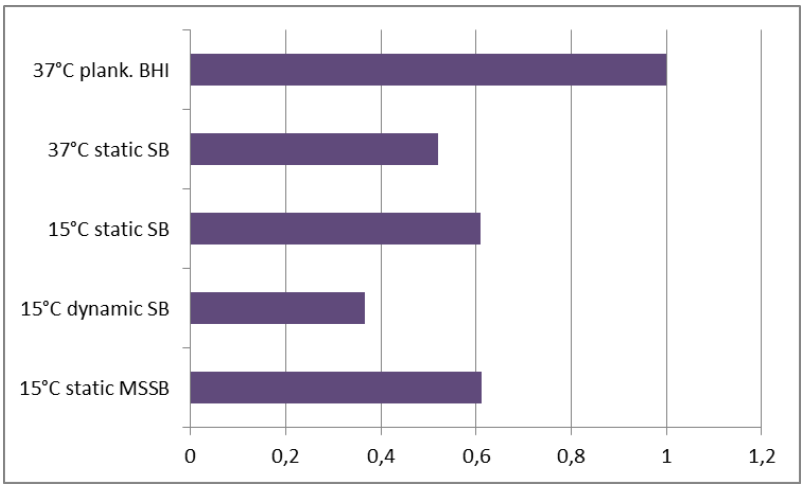


Figure 22: Expression ratio of *sigB* normalised to 37°C planktonic BHI

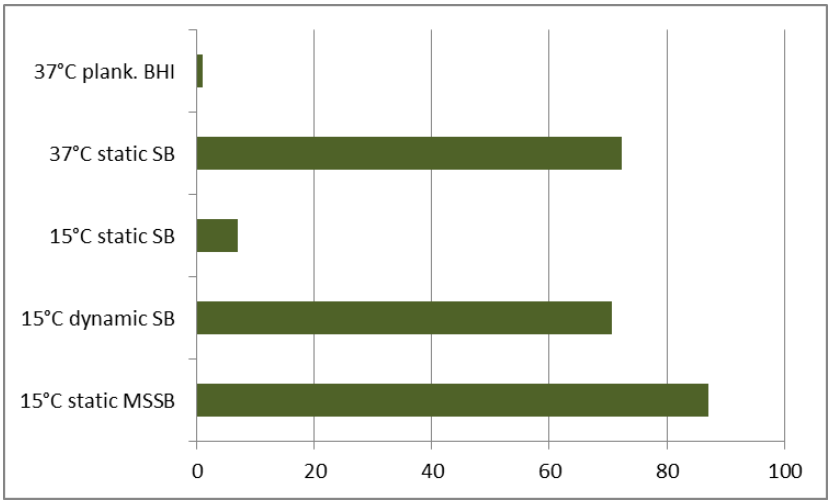


Figure 23: Expression ratio of *prfA* normalised to 37°C planktonic BHI

For *sigB* no significant change in gene expression can be seen (except for 8°C static conditions SB), while for *prfA* the gene expression is strongly upregulated for all the conditions (note the difference in magnitude of the x-axis). Since *prfA* is upregulated for all conditions and *sigB* is not, it is reasonable to assume that other than the *sigB*-dependent pathway of regulating *prfA* is activated.

To further analyse the genetic expression, a normalisation to 15°C static conditions SB pre-culture was made for 15°C dynamic conditions in SB and 15°C static conditions in MSSB. This is shown in table 8 and figure 24-25

Table 8: Expression ratio normalised to 15°C static conditions SB pre-culture

Gene/sample	15°C static SB pre-culture	15°C dynamic SB pre-culture	15°C static MSSB pre-culture
sigB	1.00	0.60	1.01
prfA	1.00	10.22	12.61

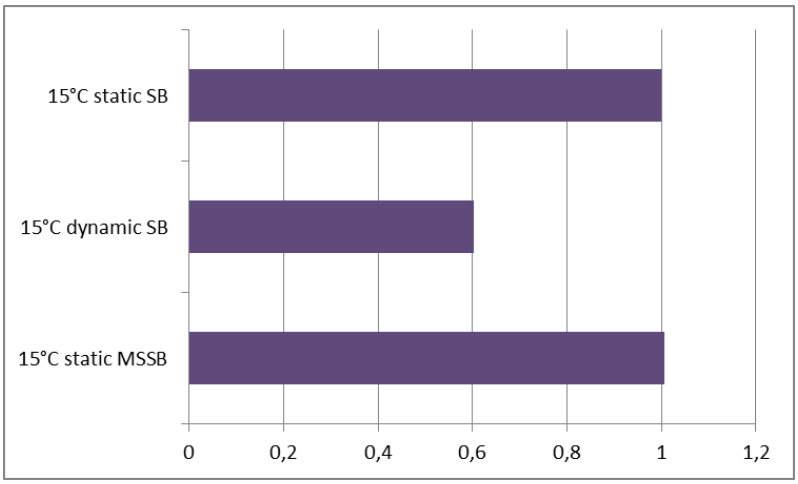


Figure 24: Expression ratio of *sigB* normalised to 15°C static SB

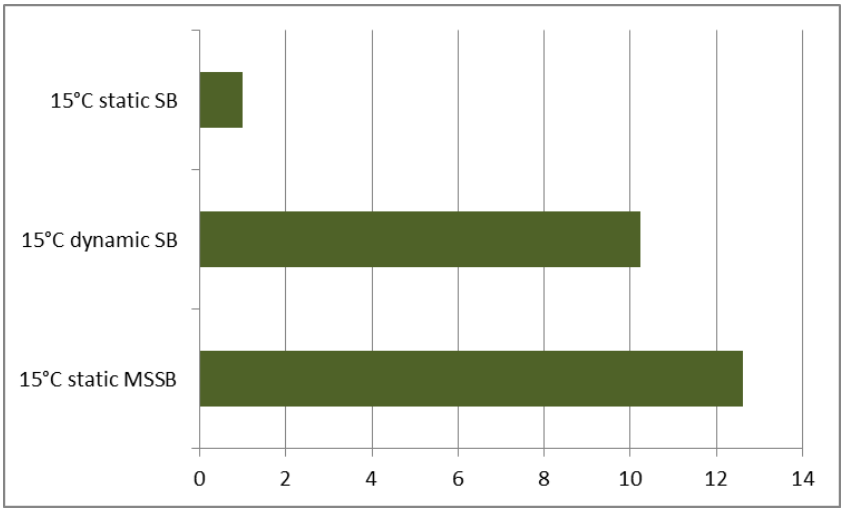


Figure 25: Expression ratio of *prfA* normalised to 15°C static SB

No significant changes in expression of *sigB* can be seen but for *prfA* the expression ratio is more than ten times that of 15°C static conditions SB for both 15°C static conditions MSSB and 15°C dynamic conditions SB.

A third normalisation is made to 37°C static conditions SB for the other temperatures of static conditions SB to compare the temperature effect and this can be seen in table 9 and figure 26-29. In figure 27 and 29 8°C static conditions SB is removed to enable the comparison of 37°C static conditions SB and 15°C static conditions SB.

Table 9: Expression ratio normalised to 37°C static conditions SB pre-culture

Gene/sample	37°C static SB pre-culture	15°C static SB pre-culture	8°C static SB pre-culture
sigB	1	1.17	97.50
prfA	1	0.10	28.37

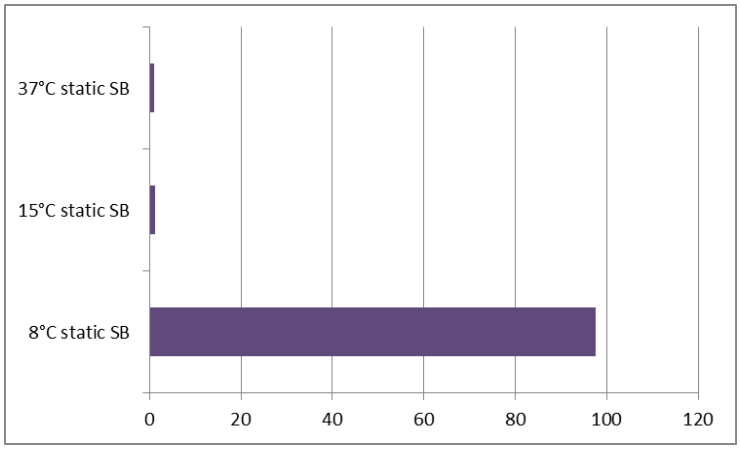


Figure 26: Expression ratio of *sigB* normalised to 37°C static SB

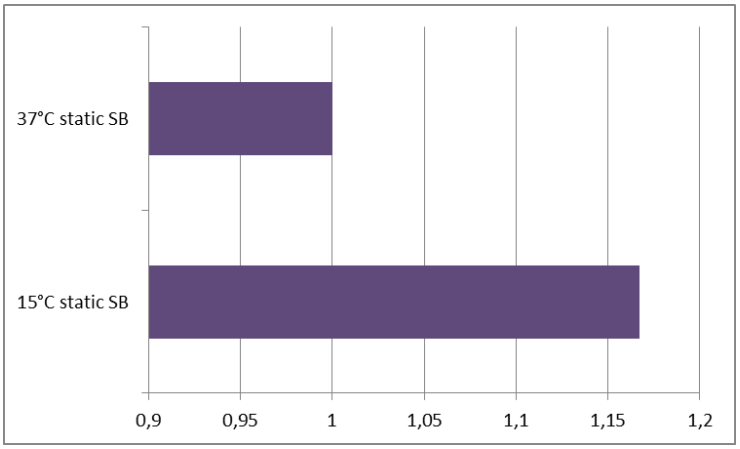


Figure 27: Expression ratio of *sigB* normalised to 37°C static SB

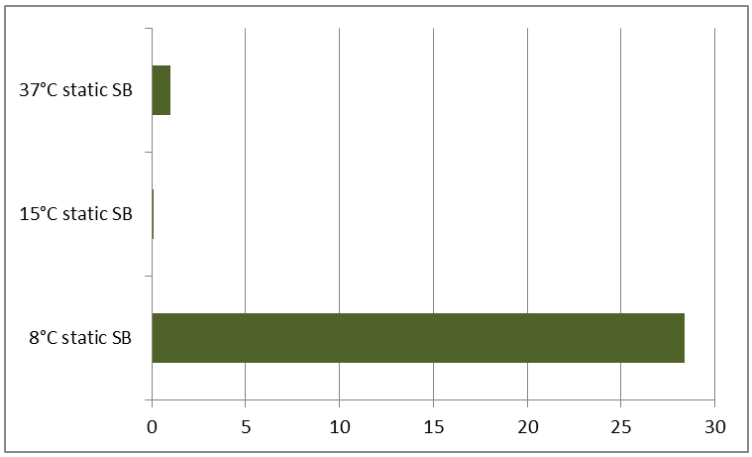


Figure 28: Expression ratio of *prfA* normalised to 37°C static SB

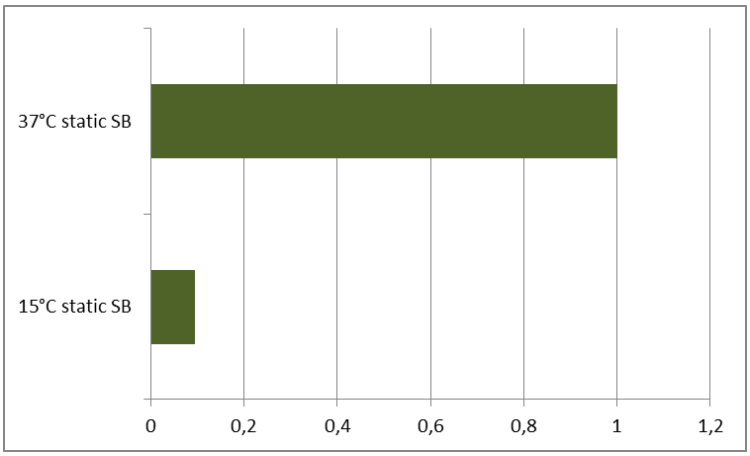


Figure 29: Expression ratio of *prfA* normalised to 37°C static SB

For *sigB* there is a significant up regulation between 8°C static conditions SB and 37°C static conditions SB but there is no significant difference between 37°C static conditions SB and 15°C static conditions SB. For *prfA* both 8°C static conditions SB and 15°C static conditions SB have changed expression but while the expression for 8°C static conditions SB is up regulated the expression for 15°C static conditions SB is down regulated compared to 37°C static conditions SB.

4 Discussion

The most pronounced result from the genetic expression analysis is the overwhelming up-regulation of *sigB* and *prfA* expression for the biofilm formed at 8°C in static conditions in SB. This is important since this implies a higher virulence from these cells, and 8°C is a normal temperature for storage in the industry and therefore it is not unlikely that contaminations could sometimes occur from biofilms growing at 8°C. For all biofilms except in 8°C static conditions in SB the expression ratio of *sigB* was unchanged but the relative expression of *prfA* was much higher for all biofilms. This indicates that in the challenge test reported here the regulation of *prfA* is to a large part *sigB* independent. Nadon *et al.* (2002) found three regulatory pathways of which one was *sigB* dependent and it seems that other factors up regulate the *prfA* expression in the biofilms, possibly *sigB* regulates *prfA* in 8°C static conditions in SB. The high expression of *prfA* is related to a higher virulence of the cells and therefore one might consider that these cells are more pathogenic than cells cultured in liquid medium.

Part from having higher gene expression of *sigB* and *prfA*, 8°C in static conditions in SB also stood out because it had no visible lag phase. This might be interpret as that the conditions in the biofilm in 8°C in static conditions in SB and on salmon are quite similar. The 37°C static conditions in SB on the other hand had a long lag time indicating that much adaptation to the new conditions had to be made. This can be compared with the results for the planktonic pre-cultures in BHI where a pre-culture in 8°C had a lag phase duration of 1.46 days and the pre-culture in 37°C had a lag phase duration of 6.77 days (Samuelsson 2012). This gives an opposite trend for the transition from planktonic BHI to biofilm in static conditions in SB, since in 8°C the lag phase is decreased from planktonic to biofilm while in 37°C the lag phase is increased.

For the different biofilms in 15°C, with the static conditions in SB as starting point, growing in dynamic conditions increased the lag phase duration on cold smoked salmon more than growing in a modified medium but both showed a heavily up-regulated expression of *prfA*. This indicates that dynamic conditions or a less nutrient rich medium increases the virulence of *L. monocytogenes*. Both these pre-cultures are just “scratching the surface” of variations that could be done within either biofilms grown dynamically or in different media and then combinations of both. Important if more challenge tests are to be conducted is that a good experimental design is constructed to evaluate the desired parameters, otherwise numerous tests will be needed since there are a lot of interesting parameters, for the medium e.g: proportion of water, SB, salmon-residue, salt, dirt etc. and for dynamic conditions; flow rate, medium volume, change of medium during the pre-culture, inoculation concentration and method etc. An experimental design generates more and statistically determined results with fewer challenge tests than “random” variations. When choosing parameters to evaluate, the conditions in industry should always be considered.

It is known that the expression of *sigB* is up regulated when cultures enter into the stationary growth phase and the 37°C planktonic BHI culture is known to be in the beginning of the stationary phase but not enough study of the biofilms were conducted to evaluate the growth phase, so it is possible that the cells from the biofilm was not in the beginning of the stationary growth phase. Thereby the expression of sigB could be unrelated to growth phase and instead correlated to stress for the biofilms. The intention during the pre-study, since the growth phase was hard to determine, was to reach a certain initial concentration on the salmon and for that the biofilm in 8°C static conditions in SB was incubated approximately three

times longer than the biofilm in 15°C static conditions in SB which might enable it to reach stationary phase even though the resulting concentration was the same. It is therefore possible that 8°C static conditions in SB could have reached the stationary phase, and respond to other stresses, with an increased *sigB* expression as consequence. This part is only speculation since the growth phase was not determined, but the possibility of an effect on gene expression from the different growth phases must be taken into account.

The lag phase duration and the growth rate was determined with DMfit and even though it can be an excellent tool it has limitation and the usefulness should be evaluated before it is applied, in this study the program could not determine a lag phase when a visual inspection of the growth curves could. Therefore it is important to examine data in more than one way to get the most comprehensive picture as possible.

One interesting attribute is that the standard deviation between the three samples is greater when the pre-culture is a biofilm than a plankton culture. This is especially evident at the end of the storage time. This implicates that more replicates and additional challenge tests must be done to generate reliable results but this large variation should not only be treated as experimental noise but must be taken into consideration when creating models or making predictions of the growth, at least if the same behaviour can be seen in future studies with more replicates at each sampling point. Perhaps this variation could partly be eliminated with further standardisations of the experimental setup but at the same time, the biofilms that might contaminate cold smoked salmon in industry cannot be considered standardise and therefore before biofilms from industry are used, some variation might serve as a margin of security. The fact that the standard deviation increases with time might imply that the cells are somewhat different in the pre-culture and with time these differences develop and thereby different growth rates can be observed. If the experimental setup was to be developed and optimised this could be a factor to take into account.

It would have been very interesting to see if this higher expression of *prfA* for the cells from the biofilms continued during growth on the cold smoked salmon and the differences between different pre-cultures at the salmon for *prfA* and *sigB* but this could not be accomplished because of the low amount of RNA in these samples. One possible explanation for this is that degrading substances from the salmon was not completely removed during RNA isolation or had already degraded some of the RNA before isolation during storage. This however might be unlikely because 1) the samples were processed with RNAprotect, design to preserve RNA expression, directly when the sample was taken and care was put into minimizing the time from removal from the storage to the outtake of the RNA sample 2) the samples were kept at -80°C both before and after RNA isolation and at this temperature no degradation should be able to occur.

The dissociation curves for *prfA* and *sigB* from the salmon samples indicates that there is no PCR-product and the Ct-values for all the genes cannot be reliable distinguished from that of the NTC, therefore the RNA isolation method must be evaluated. The measurement of RNA concentration after isolation showed various amount of RNA but there were issues with the purity. Since this was also the case for the samples where RNA had been isolated from pre-culture the analysis proceeded but it seems likely that some residue from the salmon remained in the sample after RNA isolation and interfered with the measurement. It might even be possible that for the pre-culture samples there were some contamination in the RNA sample while for the samples from the salmon, there were mostly contaminations and very little RNA.

The method of RNA isolation from salmon must be evaluated or a new method should be complied. To reduce the amount of steps one could try to isolate the RNA directly upon sampling without first freezing in RNAprotect and then proceed with reverse transcriptase and real time PCR directly. Freezing of RNA in -80°C is though a normal procedure and in itself should not affect the outcome. (This might require a two person collaboration when starting up a challenge test if inoculation of salmon, RNA isolation from pre-culture and RNA isolation from salmon are to be performed simultaneously.) The amount of *L. monocytogenes* in the sample was approximately 10^5 cfu/g and this is much lower than from a culture grown planktonic at 37°C in BHI. It should be evaluated if this is enough for isolation of bacterial RNA with this method or if the isolation actually generated salmon RNA, in that case the method must be modified and optimized for more efficient isolation of bacterial RNA. One step that was different between the procedure used in this study and Wallin-Carlquist *et al.* (2010) and that could be evaluated was that they used a BagPage with a finer filter, but the BagPage used in this study are according to the manufacturer fitting for PCR analysis.

MO-BIO (USA, California) has a kit for isolation of microbial DNA from food, PowerFood™ Microbial DNA Isolation Kit, and the use of this for RNA isolation could be considerable if some modifications are made to adjust to binding of RNA instead of DNA and steps for DNA degradation are added. Another option is to try the protocol by Rantsiou *et al.* (2008), with the modifications by Olesen *et al.* (2010) that were also used to isolate RNA from food, where MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Wisconsin, USA) was used after some sample preparations.

In addition the RT-PCR should perhaps also be optimized since there are continues problems with amplification from the NTC from *sigB* and *16S-rRNA* samples. These problems were partly addressed by Samuelsson (2012) without any successful solution and I had hoped to continue this evaluation but the time limits did not allow it. For example Udvardi (2008) or Werbouck *et al*. (2007) can be consulted as a starting point to identify possible problematic factors.

One question that might arise is if there was any expression of *sigB* or *prfA*, since it could not be detected. This would make all other explanations unnecessary. The probable answer however is yes, there should be expression of these genes. For one, the first sample from the salmon was taken within approximately one hour after inoculation with the pre-culture and especially in the case of 8°C static conditions in SB that would be a considerable change in such a limited time to enable the high expression in the pre-culture to change to no expression and no remaining mRNA on the salmon. Also, since the gene *16S-rRNA* is a housekeeping gene and should always be expressed in approximately the same amount and expression of this could neither be detected for many samples, this explanation seems very unlikely. For some samples the Ct-values for *16S-rRNA* were more acceptable (~25 cycles) but still much higher than from other conditions and there was enough *16S-rRNA* to give dissociations curves indicating pure PCR-product. Perhaps a possible RNA degradation did not proceed far enough to degrade all of the high-copy gene *16S-rRNA* but all of the low-copy genes *sigB* and *prfA*.

For further challenge test another condition that would be interesting to evaluate in this context is the storage temperature during the challenge test. It has previously been shown that low virulence stains of *L. monocytogenes* increases their virulence then stored at higher temperatures (Duodu 2010) and the recommendation for storage of cold smoked salmon is 4°C but many household as well as retailers have a higher refrigerator temperature, perhaps

8°C, and it would be interesting to see how the lag phase and gene expression is altered by this.

4.1 Further studies

The results of the genetic expression is unfortunately quite unreliable, both because the NTC for all genes gives a Ct-value close to the sample values, and because of the dissociation curves. If further studies with the same approach and aim are to be conducted, and include the genetic expression of these genes, I would recommend focusing more attention on the genetic analysis before proceeding with more challenge tests and evaluate both the RNA isolation and the RT-PCR. Since the method used for isolating RNA from salmon were successfully used by Wallin-Carlquist *et al.* (2010) for RNA isolation from meat products it ought to be adaptable for other food product such as cold smoked salmon.

With the statement from the introduction, that many factors influence the lag phase duration, it is interesting to note that today most challenge tests to determine the growth of different pathogens and spoilers on food are made with a pre-culture grown in nutrition rich medium at 37ºC. This is good for example for comparison and does give much insight in the growth of bacterium on food, but are also flawed since the lag phase will vary if the pre-culture is different than that in the challenge test. This will most defiantly be the case for bacterium actually contaminating food in the field or factory.

5. References

Arguedas-Villa, C., Stephan R., and Tasara, T. (2010) Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiology,* 27(5)**,** 653-660.

Augustin, J.C., Rosso,L., and Carlier, V. (2000) A model describing the effect of temperature history on lag time for *Listeria monocytogenes*. *International Journal of Food Microbiology,* 57(3)**,** 169-181.

Baranyi, J., and Roberts, T. A., (1994) A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology,* 23(3-4)**,** 277-294

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N. and Schmidt, G.R. (1994) Growth variation among species and strains of *Listeria* in culture broth. *Journal of Food Protection,* 57(9)**,** 765-769.

Beaufort, A. (2011) The determination of ready-to-eat foods into *Listeria monocytogenes* growth and no growth categories by challenge tests. *Food Control,* 22(9)**,** 1498-1502.

Becker, L.A., Çetin, M.S., Hutkins, R.W. and Benson, A.K. (1998) Identification of the gene encoding the alternative sigma factor σ(B) from Listera monocytogenes and its role in osmotolerance. *Journal of Bacteriology,* 180(17), 4547-4554

Becker, L.A., Evans, S.N., Hutkins, R.W. and Benson, A.K. (2000) Role of sigma(B) in adaptation of *Listeria monocytogenes* to growth at low temperature. *Journal of Bacteriology,* 182(24)**,** 7083-7087.

Belessi, C.E.A., Gounadaki, A.S., Psomas, A.N. and Skandamis, P.N. (2011) Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. *International Journal of Food Microbiology,* 145 (SUPPL. 1), S46-S52

Beumer, R.R., Te Giffel, M.C., De Boer, E. and Rombouts, F.M. (1996) Growth of *Listeria monocytogenes* on sliced cooked meat products. *Food Microbiology,* 13 (4), 333-340

Bremer, P.J., Monk, I. and Osborne, C.M. (2001) Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of Flavobacterium spp. *Journal of Food Protection,* 64(9), 1369-1376

Buchanan, R., Lindqvist R., Ross, T., Smith, M., Todd, E. and Whiting, R. (2004) *Risk assessment of Listeria monocytogenes in ready-to-eat foods Interpretative summary*. Microbiological risk assessment series 4 ©WHO / FAO 2004

Burall, L.S., Laksanalamai, P. and Datta, A.R. (2012) *Listeria monocytogenes* Mutants with Altered Growth Phenotypes at Refrigeration Temperature and High Salt Concentrations. *Applied and Environmental Microbiology,* 78(4)**,** 1265-1272.

Carpentier, B. and Cerf, O. (2011) Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology,* 145(1), 1-8

Carpentier, B. and Chassaing, D. (2004) Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. *International Journal of Food Microbiology,* 97(2), 111-122

Chakraborty, T., Leimeister-Wachter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T. and Notermans, S. (1992) Coordinate Regulation of Virulence Genes in *Listeria monocytogenes* Requires the Product of the *prfA* Gene. *Journal of Bacteriology* 174(2), 568-574

Chang, Y., Gu, W., Fischer, N. and McLandsborough, L. (2012) Identification of genes involved in *Listeria monocytogenes* biofilm formation by *mariner*-based transposon mutagenesis. *Applied Microbiology and Biotechnology,* 93(5), 2051-2062

Commission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (2005)

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annual Review of Microbiology*, 49, 711-745

Dramsi, S., Kocks, C., Forestier, C. and Cossart, P.(1993) Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA. Molecular Microbiology*, 9(5), 931-941

Dufrenne, J., Delfgou, E., Ritmeester, W. and Notermans, S. (1997) The effect of previous growth conditions on the lag phase time of some foodborne pathogenic micro-organisms. *International Journal of Food Microbiology,* 34(1), 89-94

Duodu, S., Holst-Jensen, A., Skjerdal, T., Cappelier, J.-M., Pilet, M.-F. and Loncarevic, S. (2010) Influence of storage temperature on gene expression and virulence potential of *Listeria monocytogenes* strains grown in a salmon matrix. *Food Microbiology,* 27(6), 795-801

Dykes, G.A. (2003)Behaviour of *Listeria monocytogenes* on two processed meat products as influenced by temperature or attached growth during preincubation. *Food Microbiology*, 20(1), 91-96

Ferreira, A., Sue, D., O'Byrne, C.P. and Boor, K.J. (2003) Role of *Listeria monocytogenes* σ B in survival of lethal acidic conditions and in the acquired acid tolerance response. *Applied and Environmental Microbiology,* 69(5), 2692-2698

Finelli, A., Gallant, C.V., Jarvi, K. and Burrows, L.L. (2003) Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology,* 185(9), 2700-2710

Francois, K., Valero, A., Geeraerd, A.H., Van Impe, J.F., Debevere, J., García-Gimeno, R.M., Zurera, G., and Devlieghere, F. (2007) Effect of preincubation temperature and pH on the individual cell lag phase of *Listeria monocytogenes*, cultured at refrigeration temperatures. *Food Microbioloy,* 24(1)**,** 32-43.

Gandhi, M. and Chikindas, M.L. (2007). Listeria: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology,* 113(1)**,** 1-15

Gay, M. and Cerf, O. (1997) Significance of temperature and preincubation temperature on survival of *Listeria monocytogenes* at pH 4.8. *Letters in Applied Microbiology,* 25(4), 257-260

Geornaras, I., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C. and Sofos, J.N. (2006) Post-processing application of chemical solutions for control of *Listeria monocytogenes*, cultured under different conditions, on commercial smoked sausage formulated with and without potassium lactate–sodium diacetate. *Food Microbiology,* 23(8), 762-771

Holah, J.T., Bird, J. and Hall, K.E. (2004) The microbial ecology of high-risk, chilled food factories; evidence for persistent *Listeria* spp. and *Escherichia coli* strains. *Journal of Applied Microbiology*, 97(1), 68-77

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J. and Wiedmann, M. (2003) *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *Journal of Bacteriology,* 185(19)**,** 5722-5734.

Leimeister-Wächter, M., Haffner, C., Domann, E., Goebel, W. and Chakraborty, T. (1990) Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes. Proceedings of the National Academy of Sciences of the United States of America*, 87(21), 8336-8340

Lelièvre, C., Legentilhomme, P., Gaucher, C., Legrand, J., Faille, C. and Bénézech, T. (2002) Cleaning in place: effect of local wall shear stress variation on bacterial removal from stainless steel equipment. *Chemical Engineering Science*. 57(8), 1287-1297

Lemon, K.P., Freitag, N.E. and Kolter, R. (2010) The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *Journal of Bacteriology*. 192(15), 3969-3976

Lövenklev, M., Artin, I., Hagberg, O., Borch, E., Holst, E. and Rådström, P. (2004) Quantitative interaction effects of carbon dioxide, sodium chloride, and sodium nitrite on neurotoxin gene expression in nonproteolytic *Clostridium botulinum* type B. *Applied and Environmental Microbiology*, 70(5), 2928-2934

Marron, L., Emerson, N., Gahan, C.G.M. and Hill, C. (1997) A mutant of *Listeria monocytogenes* LO28 unable to induce an acid tolerance response displays diminished virulence in a murine model. *Applied and Environmental Microbiology*, 63(12), 4945-4947

Mengaud, J., Dramsi, S., Gouin, E., Vazquez-Boland, J.A., Milon, G. and Cossart, P. (1991)Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. *Molecular Microbiology*, 5(9)**,** 2273-2283.

Midelet, G. and Carpentier, B. (2002) Transfer of microorganisms, including *Listeria monocytogenes*, from various materials to beef. *Applied and Environmental Microbiology*. 68(8), 4015-4024

Montwille, T. and K. Matthews (2008) *Food Microbiology : An Introduction*. Second ed, Washington DC, USA, AMS Press

Moorhead, S.M. and Dykes, G.A. (2003) Influence of the sigB gene on the cold stress survival and subsequent recovery of two *Listeria monocytogenes* serotypes. *Current Microbiology*, 46, 461-466

Nadon, C.A., Bowen, B.M., Wiedmann, M. and Boor, K.J. (2002) Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. *Infection and Immunity*, 70(7), 3948-3952

Nilsson, C. and Lindblad, M. (2011) *Listeria monocytogenes i kyld och ätfärdig mat.* Riksprojekt 2010. National food administration, Sweden

Norwood, D.E. and Gilmour, A. (2001) The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Letters in Applied Microbiology*. 33, 320-324

Notermans, S., Soentoro, P.S.S., Bolder, N.M. and Mulder, R.W.A.W. (1991) Adaptation of Listeria in liquid egg containing sucrose resulting in survival and outgrowth. *International Journal of Food Microbiology*, 13(1), 55-62

O'Driscoll, B., Gahan, C.G.M. and Hill, C. (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. *Applied and Environmental Microbiology*, 62(5), 1693-1698

Oh, Y.J., Jo, W., Yang, Y. and Park, S. (2007) Influence of culture conditions on Escherichia coli O157:H7 biofilm formation by atomic force microscopy. *Ultramicroscopy*, 107(10-11), 869-874

Olesen, I., Thorsen, L. and Jespersen, L. (2010) Relative transcription of *Listeria monocytogenes* virulence genes in liver pates with varying NaCl content. *International Journal of Food Microbiology*, 141(SUPPL.), S60-S68

Olesen, I., Vogensen, F.K. and Jespersen, L. (2009) Gene transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathog Disease* 6(6), 669-680

Pfaffl, M.W (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*, 29(9)**,** e45

Poimenidou, S., Belessi, C.A., Giaouris, E.D., Gounadaki, A.S., Nychas, G.-J.E. and Skandamis, P.N. (2009) *Listeria monocytogenes* attachment to and detachment from

stainless steel surfaces in a simulated dairy processing environment. *Applied and Environmental Microbiology*, 75(22), 7182-7188

Potera, C. (1998) Studying slime. *Environmental Health Perspectives*, 106(12), A604-A606

QIAGEN (2005) RNAprotect Bacterial Reagent Handbook. 2005 [Manual, accessed online mars 2012]

Rantsiou, K., Alessandria, V., Urso, R., Dolci, P. and Cocolin, L. (2008) Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *International Journal of Food Microbiology*, 121(1), 99-105

Rodrigues, D., Cerca, N., Teixeira, P., Oliveira, R., Ceri, H. and Azeredo, J. (2011) *Listeria monocytogenes* and *Salmonella enterica* Enteritidis biofilms susceptibility to different disinfectants and stress-response and virulence gene expression of surviving cells. *Microbial Drug Resistance*, 17(2), 181-189

Samuelsson, C. (2012) *The influence of different stress factors on the lag phase and genetic expression of* Listeria monocytogenes *in cold smoked salmon*. Master thesis at Linköping University

Shetron-Rama, L.M., Mueller, K., Bravo, J.M., Bouwer, H.G.A., Way, S.S. and Freitag, N.E. (2003) Isolation of *Listeria monocytogenes* mutants with high-level in vitro expression of host cytosol-induced gene products. *Molecular Microbiology*, 48(6), 1537-1551

Simões, M., Simões, L.C., Machado, I., Pereira, M.O. and Vieira, M.J. (2006) Control of flow-generated biofilms with surfactants: Evidence of resistance and recovery. *Food and Bioproducts Processing*, 84(4C), 338-345

Simões, M., Pereira, M.O., Sillankorva, S., Azeredo, J. and Vieira, M.J. (2007) The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms. *Biofouling*, 23(4), 249-258

Smittskyddsinstituet (2012) http://www.smittskyddsinstitutet.se/ (22 Augusti 2012)

Udvardi, M.K., Czechowski, T. and Scheible, W.-R. (2008) Eleven golden rules of quantitative RT-PCR. *Plant Cell*,20(7), 1736-1737

van der Veen, S. and Abee, T. (2010) Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. *Applied and Environmental Microbiology*, 76(23)**,** 7854-7860

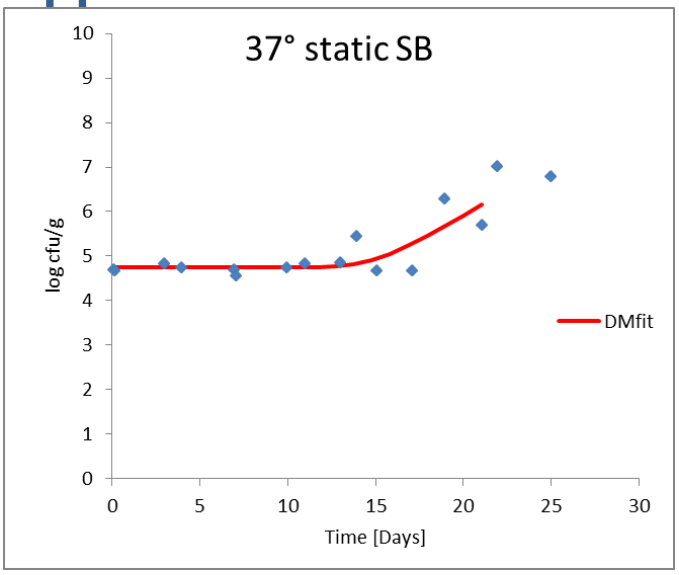
Wallin-Carlquist, N., Márta, D., Borch, E. and Rådström, P. (2010) Prolonged expression and production of Staphylococcus aureus enterotoxin A in processed pork meat. *International Journal of Food Microbiology*, 141(SUPPL.), S69-S74

Werbrouck, H., Botteldoorn, N., Uyttendaele, M., Herman, L. and Van Coillie, E. (2007) Quantification of gene expression of *Listeria monocytogenes* by real-time reverse transcription PCR: optimization, evaluation and pitfalls. *Journal of Microbiological Methods*, 69(2)**,** 306-314

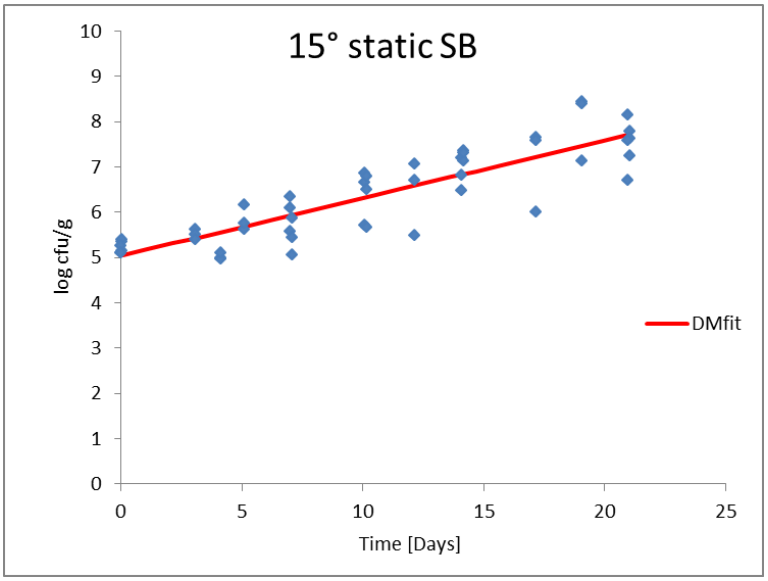
Wiedmann, M., Arvik, T.J., Hurley, R.J. and Boor, K.J. (1998) General stress transcription factor δ(B) and its role in acid tolerance and virulence of *Listeria monocytogenes.* *Journal of Bacteriology*, 180(14), 3650-3656

Wirtanen, G. (1995), *Biofilm formation and its elimination from food processing equipment*, PhD thesis, VTT Publications 251, Esbo

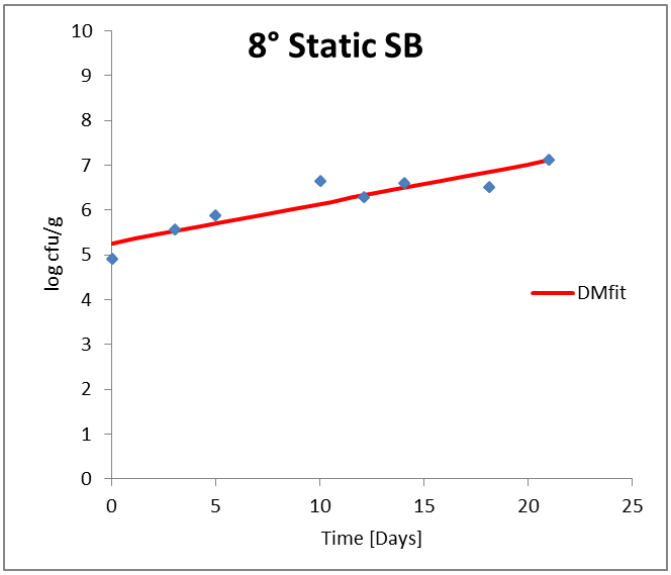
Appendix I



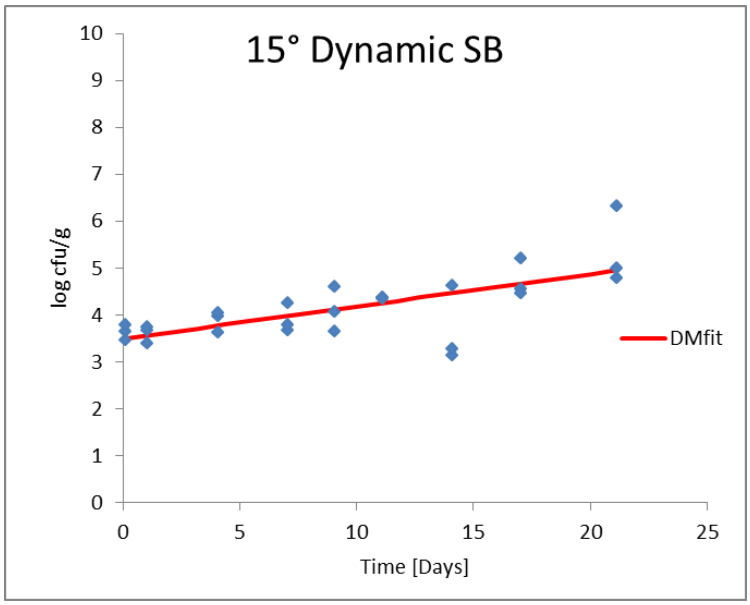
App figure 1: DMfit for 37°C static SB with mean values



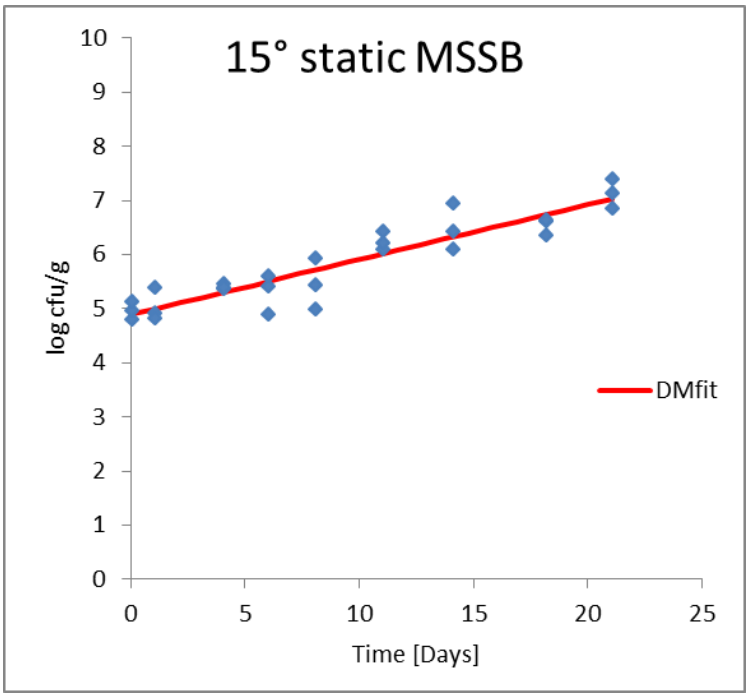
App figure 2: DMfit for 15°C static SB with all values



App figure 3: DMfit for 8°C static SB with mean values



App figure 4: DMfit for 15°C dynamic SB with all values



App figure 5: DMfit for 15°C static MSSB with all values

Appendix II

Isolate RNA without a “commercial kit”
Modified from Lövenklev et al (2004)

1. Put the salmon sample in a BagPage filter bag and add 9 parts (weight) of Buffered peptone water
2. Run stomacher 40s
3. Take a sample of 5 ml from the slurry to a centrifuge tube containing 10ml RNAprotect.
4. Vortex and then incubate for 5 min at room temperature
5. Centrifuge at 5000xg, 10 min at 4°C
6. Remove supernatant and store pellet in -80°C until further processing
7. Resuspend the pellet in 400µl ice cold TES buffer[1]
8. Prepare a 2 ml tube containing 1ml 0,1mm silica beads, 600µl acid phenol and 100µl chlorophorm.
9. Add the cell suspension to the 2 ml tube and put the tube in the Mini Bead Beater. Shake the tube for 45s, chill on ice for 30s and then shake the tube for additional 45s.
10. Chill the tube on ice
11. Centrifuge the tube 5000rpm, 5min (4°C)
12. Transfer the upper phase (water phase) to a new tube (eg. Ependorf). Take the same volume from each tube for high reproducibility
13. Add phenol:chlorophorm (600µl:100µl) and vortex
14. Centrifuge 14 000rpm, 5 min
15. Take the water phase to a new tube and repeat extraction with phenol:chlorophorm
16. Add 600µl chlorophorm and vortex
17. Centrifuge 14 000rpm 5min
18. Remove the upper (water) phase, approximately 200µl
19. Add 1/10 volume of 3M NaAc, pH 4.8 and 2.5 volumes of 95% ethanol
20. Incubate at -70°C for 20min to precipitate the RNA, or longer at -20°C
21. Centrifuge 14 000rpm 20 min 4°C

22. Remove/discard supernatant
23. Dry upside down on kleneex
24. Wash with 600µl 70% ethanol
25. Centrifuge 14 000rpm 10min
26. Dry upside down on kleneex
27. Resuspend in 200µl DEPC water
28. Add 20µl RDD buffer and 5 µl DNase I stock solution Not: used 40 and 10 the first two times
29. Incubate at 37°C for 45 minutes
30. Add 800µl of phenol:chloroform:isoamyl alcohol (pH 6.5 – 8.0) and vortex to mix
31. Incubate at room temperature for 5 minutes.
32. Centrifuge the sample at 10,000 x g for 5 minutes.
33. Carefully remove the upper aqueous phase and transfer it to another tube.
34. Add 1/10th volume of 5M NaCl, two volumes of 100% ethanol and invert to mix.
35. Incubate at -20°C for 30 minutes and centrifuge at 10,000 x g for 10 minutes.
36. Decant the supernatant and air dry the pellet.
37. Resuspend the pellet in 50 µl DEPC water
38. Freeze in -80°C until analysis

Material

[1]TES buffer: 3,03g 50mM Tris 0,303g
0,93g 5mM EDTA 0,093
1,45g 50Mm NaCl 0,145
500ml water, adjust pH to 7.5 50ml

3M NaAc + 3M acetic acid
81,6g in 200ml DEPC water 8,16g in 20ml
3M acetic acid: took 19ml of >90% acetic acid and 81ml dest water